STRUCTURAL STUDIES ON SOME PLANT CONSTITUENTS

A THESIS

Submitted for the Degree of

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BY

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DEDICATED

TO

MY PARENTS

PREFACE

The present thesis entitled, "STRUCTURAL STUDIES ON SOME PLANT CONSTITUENTS" embodies the results of chemical investigation of the whole plant of Cassia occidentalis and the fruit pericarp of Feronia limonia .

All the work described in this thesis has been carried out, in the Natural Products Laboratory of Department of Chemistry, University of Allahabad, Allahabad, India, by me under kind supervision of Dr. Jagdamba Singh, Reader, Department of Chemistry, University of Allahabad, Allahabad, India.

The subject matter of the thesis has been divided into three chapters. Chapter -1 reviews the past chemical work done on Cassia occidentalis and Feronia limonia. This chapter throws light on the morphology and medicinal value of these plant along with present work. A brief description of coumarins, flavonoids, triterpenoids and physio-chemical techniques employed for structure elucidation of triterpenoids is also given in this chapter.

Chapter-2 deals with isolation, purification and identification of the compounds, from the plant of Cassia occidentalis. This chapter is further divided into 3 sections which deals with the detailed structural studies of the chemicals constituents isolated from the plant of Cassia occidentalis.

Chapter-3 incorporates detailed chemical investigation of the pericarp of Feronia limonia. Detailed structural studies of the compounds isolated from this species has been given under section I, II, III, IV of this chapter, respectively.

An abstract of the entire work incorporated in this thesis is being submitted separately along with it as required by the Ordinances for the D.Phil degree of University of Allahabad.

Chhavi Purwar

(Chhavi Purwar)

ACKNOWLEDGEMENT

Without believe in co-operation

Look what happens to a wagon

that looses a wheel

Napoleon Hill

I unhesitatingly acknowledge that this work would have not been completed without the help, inspiration and guidance of my colleagues, academic friends and teachers. I deem it an honour and privilage to express my sincere gratitude to my scintillating guide and mentor Dr Jagdamba Singh whose enduring guidence and sincere encouragement at every stage of my work.

I express my gratitude to Prof K. P. Tiwari, Head, Department of Chemistry, University of Allahabad, Allahabad for providing the necessary research facilities. I am also thankful to Prof J. D. Pandey, former Head, Department of Chemistry, University of Allahabad, Allahabad for his help at every stage of my research work.

I am extremely thankful to Dr I. R. Siddiqui for his help in a wide variety of ways by providing invaluable suggestions during the course of my research work.

My sincere thanks to senior research colleagues Dr Snehlata Tripathi, Dr Anjali Gupta, Dr Aditi Gupta, Dr Renu Rai, Niharika and Dr Deepa Chauhan for their enormous

encouragement, interest, indispensable assistance and advice helped me throughout my research work from their wealth of knowledge and experience.

I am beholden to my research colleagues Mr Rakesh Misra and Mrs Rachana Singh and friends Miss Manjari Govil and Miss Meenakshi Pandey for their co-operation and enlighted me in so many way in promoting my research work.

My affection goes to junior research colleagues Mrs Deepali Rai, Miss Vibha Singh, Miss Nidhi Srivastava, Mr Chandra Prakash Sharma, Miss Pushpa and Miss Neetu Chaurasia for their company.

I would like to extend my gratitude to University of Glasgow, U. K. and R.S.I.C., CDRI, Lucknow for spectral analysis.

I am indebted to Botanical Survey of India, Allahabad and Botany Department, University of Allahabad, Allahabad that helped me in identification of plants.

Vijay and Moolchand will always be remembered with thanks by me for their prompt assistance in all my laboratory works.

Finally I thank the members of my family for their support and encouragement in my work.

To all I am highly obliged.

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CONTENTS

CHAPTER - 1

INTRODUCTION

Human beings have been dependent on higher plants for their health care needs since the very beginning of human civilization. In addition to food, clothing and shelter, the green plants have provided all the medicaments to man and domestic animals for thousands of years. Initially the plants were the main part of folk medicine practised by the ancient man in different parts of the world which include India, China, Middle East, Africa and South America. The folk medicine in various countries gave rise to traditional systems of medicine. Some of the most important systems still practised in the third world countries especially Unani practised in India, Pakistan, Afghanistan, Bangladesh and the Middle East, Ayurveda and Siddha practised in India, Nepal, Bangladesh and Sri Lanka and the Chinese system of medicine employed in China and other far eastern countries derive more than 80% of therapeutic agents from plants.

From folk medicine and traditional system of medicine, medicinal plants were adopted into modern system of medicine after they have been found effective drugs through chemical and pharmacological screening. In the initial stages of development of modern medicine plants and plant products formed an important part of pharmacopoeia. However, because of significant development in synthetic drug chemistry and antibiotics, there was certain amount of decline in the use of plants in modern medicine and at one time one would have thought that ultimately chemists will be able to synthesise all the active constituents of plants which are required by the modern medicine. Because of the realisation of health hazards

and toxicity associated with the indiscriminate use of synthetic drugs and antibiotics, there has been considerable resurgence in the use of herbs and herbal drugs all over the world and especially in the U.S.A. and Europe. As modern medicine has not been able to provide cure to some of the diseases like cancer, AIDS and arthritis, the future of mankind is partially dependent for their health care needs to the flora of the tropical rain forests.

The medicinal value of drug plants is due to presence of some chemical substances in the plant tissue which produce a definite physiological action on the human body. The most important chemical substances are alkaloids, terpenes, sterols, glycosides, saponins, gums, fatty acids, lactones, coumarins, carbohydrates, essential oils, waxes, amino acids, proteins, tanins, enzymes, resins, hydrocarbons, colouring matters, aliphatic ketones, esters and alcohols etc.

Reserpine, an alkaloid from *Rauwolfia sp* has been used in treating anxiety, tension, insomnia and hypertension. The medicinal value of cinchona bark was found to be due to the presence of certain alkaloids, viz. quinine, cinchonine, quinidine The antimalarial activity is due to presence of these alkaloids.

The work embodied in this thesis involves the detailed chemical investigation of two plants:

(1) Whole plant of *Cassia occidentalis*

(2) Fruit pericarp of *Feronia limonia*

The plants are well known for their medicinal importance. The main objective of this investigation was to study the chemical constituents of these plants responsible for their astringency and medicinal value. The chemical investigation of the two plants includes isolation and structural elucidation of different chemical constituents with the help of chemical reactions and spectral studies viz. UV, IR, ^{1}H NMR, ^{13}C NMR and Mass.

A brief description of these plants and their medicinal value and past literature survey of the chemical work done, along with a brief resume of the present work has been discussed in this chapter.

Cassia occidentalis

Cassia occidentalis (Syn. *Senna occidentalis*) belongs to family Leguminosae and subfamily Caesalpiniaceae commonly known as "Kasondi" in Hindi.[1] The plant is generally distributed throughout India and the tropics[2].

The plant is annual shurb. This plant is reported to have many medicinal properties.[2] The whole plant is purgative, tonic febrifuge and used in native medicins. The plant is considered a cure for sore eyes (Hughes-Buller) and enters into the composition of numerous ointments prescribed in skin diseases[3].

The root is useful in ringworm, elephantiasis, scorpion-sting, snake bite, cures ascities, heals wounds. The root is considered bitter, tonic, stomachic and have diuretic properties.[3] The root is to be beneficial in obstructions of the stomach and in incipient dropsy. The

bark of roots is used as quinine to cure fever and the juice squeezed into the nose to cure headach. American Indian use the fresh root as an antidote against various poisons.

The leaves are tasty; aphrodisiac, alexeteric; cure cough, hiccough, asthma, "kapha" and "vata"; sweetish bitter, stomachic; cure "tridosha" fevers; good for sore throat and biliousness (Ayurveda)[3]. The leaves are depurant and febrifuge. The warm baths, which are given for all disorders, have a quantity of the leaves thrown into them. This is reliable remedy for the cure of rheumatism and in all fever cases the bodies of the patients are rubbed with them. The fresh leaves are ground and applied to wounds and swellings; boiled are used in lotion or fumigation. As a cure for guinea worm, the leaves are pounded with salt and onions and applied to affected parts. The leaves are used to cure fever in children and are also squeezed in the eyes of oldmen and children to cure eye complaints.

The fruit is a cure for scorpion-sting.[3] The seeds are bitter, they are used in heat of the blood, for winter cough and for cough in animals. (Yunani).

The seeds and leaves are used externally in cutaneous diseases and have antiperiodic value. The seeds are tonic, febrifuge and used as a cure for convulsions of children. The roasted seeds is an excellant diuretic and also used as a blood tonic[3].

PAST WORK

On reviewing the literature it was found that from this plant following compounds are reported.

The protein keto fatty acid 7-oxo-11-octadecenoic acid[4], oxymethylanthraquinones[5], tannic acid, mucilage, fatty oil[6,7] toxal bumin[5], chrysarobin[8,9], galactomannan[10,11] isolated from extract of seeds. Pinselin, 1,7-dihydroxy-3-methyl xanthones, 1,8-dihydroxyanthraquinone, 12-hydroxyanthraquinone[13,14,15], phytosterol, emodin, quercetin and substance similar to rhein has been isolated from roots of *Cassia occidentalis*. Anthraquinone glycosides flavonoids[16] bianthraquinone, chrysophanol[17,18] dianthronic heteroside[19], emodin[18], physcion isolated from leaves of this plant. Chryosphanol and emodol from young roots, C-flavonosides of apigenin[19] from pericarp, flavonoid glycosides from pods[20], have also been reported. An alkaloid lycorine[21] which have inhibitory effect on tumour cells, has been determined from this plant.

Feronia limonia

Feronia limonia swingle (syn. *F. elephantum* correa) is a common Indian tree belongs to the tribe Citreae and subtribe Balsamocitrinae[22]. It is commonly known as "Kaitha" in Hindi[23], wood apple or elephant apple in English and as 'Kapitha' in Sanskrit belongs to the family Rutaceae. The Plant is a native of India and Ceylon and is found throughout the plains of India[24,25], particularly in dry situations. It occurs, wild or cultivated, upto an elevation of 1,500ft. in Western Himalayas.

Feronia is a single speciesed genus. The plant is small deciduous tree. It is moderate sized tree armed with straight sharp strong spine, 1.2-1.3 cm long. Two types of the plant are recognised,

one with small acidic fruits and the other with large sweet ones. The pulp of the ripe fruit is edible. The fruit is 5-6.3 cm in diameter, globose hard, pericarp woody, rough grey coloured, seeds numerous, small, compressed embedded in a sweetish aromatic edible pulp.

The plant is well known for its medicinal properties. The fruit is aromatic, sour-sweet, refrigerant, cardiotonic, astringent, diuretic tonic to the liver and lungs, good for throat, binding, antiscorbutic, alexiformic. It is also used in treatment of diarrhoea, dysentry, leucorrheoa, consumption, vomiting, blood impurities, fatigue, thirst, hiccough, cures cough, asthma, tumours, ophthalmia, gums strengthening, stomachic and stimulant in disease of children. The juice of fruit is good for stomatitis, earache, sore throat, useful in biliousness, topically it relieves the pain due to stings of wasps and other insects.

The unripe fruit is alexipharmic, astringent to the bowels, removes itching of the body, increases "vata", "pitta", and "kapha" and used in combination with bela and other medicines in diarrhoea and dysentry.

The pulp is used for affections of the gum and throat. The pulp applied externally is a remedy for the bites of venomous insects, if not obtainable, the powdered rind may be used.

The seeds cure heart diseases, headache, an antidote to poisons, the oil is acrid, astringent, alexiteric, stops hiccough and vomiting, cures rate bite and all poisonings destroys biliousness.

The flowers are an andidote to poisons. The leaves are aromatic, carminative, astringent and good for vomiting, hiccough, dysentry and slight bowel affections of childern.

The bark is occasionally prescribed for biliousness. In combodia, the thorns are used as a styptic in metorrhagia and the bark is a remedy for the bites and stings of venomous insects.

The roots are prescribed in the treatment of snake-bite. The fruit, root, bark and leaf are prescribed in the treatment of snake-bite (Charaka, Sushruta, Vagbhata). The fruit is recommended in scorpion sting (Charaka, Sushruta)

No part of the plant is an antidote to snake venom (Mhaskar and Caius). The fruit is useless in the treatment of scorpion sting (Caius and Mhaskar).

The tree exudes a gum from the trunk and branches, resembling gum arabic in properties. The tree is lopped for fodder in U.P. and Bombay.

PAST WORK

The different group of workers have reported the following compounds from various parts of the plant., *F. limonia.*

The presence of stigmasterol has been reported from the unripe fruit of this plant[26]. Three components described as having characteristic wood apple aroma are methyl hexanoate, ethyl-3-hydroxy hexanoate and butanoic acid[27].

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Alkaloids, coumarins, fatty acids and sterols were determined in fruit pericarp extraction of *F. Limonia*. The presence of umbelliferone, dictamnine, xanthotoxol, scoparone, xanthotoxin, isopimpinellin, isoimperatorin and marin has been reported.[28]

The defatted seeds contain proteins, carbohydrates[29], fatty acids[30] and lipids.

The leaves were reported to contain stigmasterol, psoralen, bergapten, orientin, vitexin and saponarin[31]. A compound p-methoxy benzoic acid (Anisic acid) a minor constituent from the essential oil of *F. limonia* leaves has been reported. The presence of β-amyrin, lupeol, psoralen, bergapten, xanthotoxin, umbelliferone and marmesin, in addition to C-flavonoid glycoside, isovitexin, saponarin, vitexin and orientin. Gas chromatography analysis of the unsaponifiable matter of the leaves revealed the presence of a series of $C_{16} - C_{32}$, cholestrol, campesterol, stigmasterol and β-sitosterol[33]. The fatty acids palmitic, linoleic, oleic, linolenic and stearic acids were identified in the saponifiable fraction.

In the stem bark of *F. limonia,* the presence of two uncharacterized alkaloids have been reported[26]. From trunk bark fernolin has also been reported[34]. From the heart wood of *F. limonia,* urosolic acid and 7-methylporiol-β-*D*-xylopyranosyl-*D*-glucopyranoside have been reported[35].

The roots of *F. limonia* contain geranyl, umbelliferone, osthol, isopimpinellin, bergapten and marmin[36]. A new monoterpenoid 5-methoxyfuranocoumarin lactone, named as Fernolin[37] and

aurapten, marmesin, and xanthotoxin were isolated from the roots of *F. limonia.* Furanocoumarin show photosensitizing effects[38] and xanthotoxin and bergapten are used for the treatment of leucoderma, characteristic of vitiligo[39].

Essential oils from *F. limonia* exhibited antibacterial activity[40].

OBJECTIVE AND BRIEF RESUME OF PRESENT WORK.

The main objective of the present work was to study chemical constituents present in these plants, which may be responsible for their pharmacological activity.

The procedures generally used for the isolation of the compounds were column chromatography and preparative thin layer chromatography. Structure of the compounds isolated, was established mainly on the basis of spectral evidences i.e. UV, IR, ^{1}H NMR, Mass and ^{13}C NMR.

The compounds isolated are listed below.

***Cassia occidentalis* (Whole plant)**

1. 3,2'-dihydroxy-7,8,4'-trimethoxyflavone-5-*O*-{–*β*-*D*-glucopyranosyl (1→2)}-*β*-*D*-galactopyranoside.
2. Apigenin-7-*O*-*β*-*D*-allopyranoside
3. 2*α*, 3*β*, 19*α*, 23-tetrahydroxy-urs-12-en-28-oic acid

Compound 1 and 2 were found to be new and have not been reported earlier from any other plant sources while compound 3 has been reported earlier from other plant sources.

Feronia limonia **(pericarp of fruit)**

1. 2'-hydroxyisopropyldihydrofuranocoumarin

2. Fernolin

3. 7-geranyloxycoumarin

4. Feronolide

Compound 1, 2, 3 and 4 are also known from some other plant sources.

REVIEW ON FLAVONOIDS

During the present work a number of compounds have been isolated from the whole plant of *Cassia occidentalis* and the fruit pericarp of *Feronia limonia*. From the whole plant of *Cassia occidentalis* and the fruit pericarp of *F. limonia* compounds isolated have been found to belong to flavonoid group of the naturally occuring plant pigments.

A brief resume of the chemistry of this pigment including the method of extraction, isolation and structural elucidation has been given below.

Flavone and flavonols

The term flavonoid covers a large number of naturally occuring phenolic compounds which are grouped together as chalcones, dihydrochalcones, aurones, flavanones, flavones and isoflavones, 2,3-dihydroflavonols, flavan-3,4-diols, anthocyanidins and catechins.

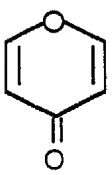
The flavones, which are also known as anthoxanthins, are widely distributed yellow plant pigments. They occur either in free state or as glycosides or associated with tannins. The anthoxanthins also occur as colourless glycosides in the white corollas of several flowers which on treatment with ammonia vapour turns yellow.

The name flavone for the unknown 2-phenyl benzopyrons (I) was first suggested by Von Kostanecki[41] and Tambor. The systematic

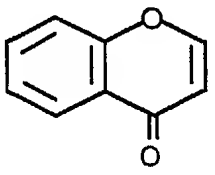
investigations of natural flavones began with the studies initiated by Kostanecki, Herzig and Perkin.

Basic structures of flavones and flavonols

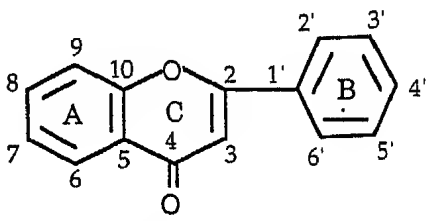
The basic unit of flavones and flavonols is ϒ-pyrone, which is present as benzo-ϒ-pyrone (chromone). The benzo-ϒ-pyrone is substituted by a phenyl group in position 2 to give the first member (flavone) of the class flavones. When hydrogen atom on C_3 in the ϒ-pyrone ring of flavone is replaced by a hydroxyl group, 3-hydroxy flavone or flavonol is produced which is the first member of the class flavonols.



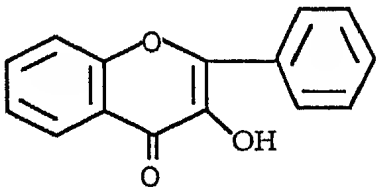
ϒ – pyrone



benzo-ϒ-pyrone



2-phenyl benzo- ϒ-pyrone or flavone (I)

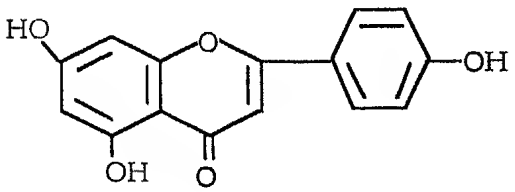


3-hydroxyflavone or flavonol (II)

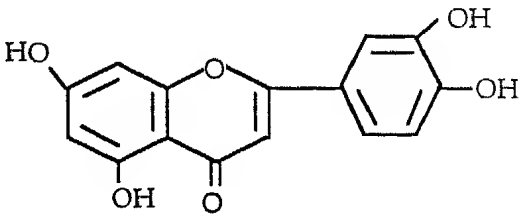
The various flavones and flavonols are hydroxy derivative of the flavone (I) and the flavonol (II), respectively.

The parent unsubstituted flavone, produced apparently by an aberrant biosynthetic pathway, occurs in the farina on species of

primula and the closely related Dionsia (Harborne 1971)[42]. The A-ring of the great majority of flavones is derived from phloroglucinol and B-ring is oxygenated in the 4' - or 3',4' - or 3', 4', 5' - positions as expected from their established Acetate - Shikimate biosynthetic origin. Apigenin (A) and luteolin (B) free and as glycosides, are the most widely occuring flavones.

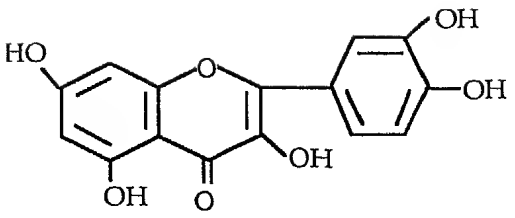


(A)

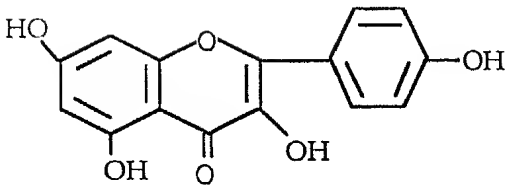


(B)

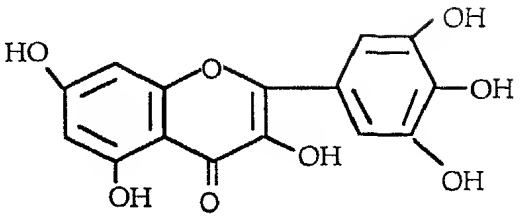
A vast range of different flavone and flavonol glycosides have now been reported in plants. For quercetin, the most common flavonol aglycone, over seventy glycosidic combinations have been fully characterised and many more have been partly analysed. Quercetin (C) must thus be unique among all the many known natural plant constituents occuring in quite so many different combined forms. Almost as many glycosides have been described in the case of the other two common flavonols, Kaempferol (D) and myricetin (E) and there are also numerous derivatives of the two common flavones, apigenin and luteolin.



(C)



(D)



(E)

Colour tests

For preliminary identification of the flavonoid compounds, colour tests are used. On treatment with magnesium and hydrochloric[43] acid pink to red colour is given by flavones and flavonols while isoflavones give yellow colour. The colour tests are only broad indications, because within a given class the colour produced depends on the hydroxylation and related substitution pattern. Flavones containing a 3-hydroxyl group give yellow colour with alkali changing to brown by oxidation in air. Flavones with 5,6,7 or 3', 4' ,5'- trihydroxyl groups give yellow colour turning deep with greenish precipitate.

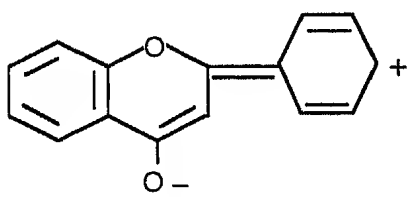
The number of hydroxyl groups in a flavonoid compound is indicated by its solubility in water, aqueous sodium carbonate or sodium hydroxide. Acidic character of flavonoids is increased due to a hydroxyl group para to the carbonyl group. Polymethoxy

compounds dissolve in concentrated hydrochloric acid with an intense yellow colour and often give precipitate with alkaloid reagents[44]. The presence of a 5-hydroxy group is shown by orange to red colouration with ferric chloride, and a bathochromic shift on adding aluminium chloride. Basic lead acetate gives coloured precipitate with most of the flavonoid polyphenols.

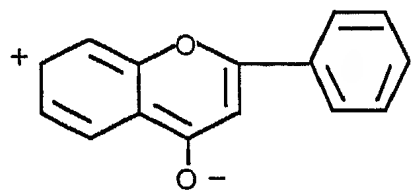
Spectral studies

UV spectra

Generally flavones and flavonols exhibit absorption in 320-380 nm region (Band I) and 240-270 nm region (Band II). In general terms, the Band II absorption may be considered as having originated from A-ring benzoyl system (XIII) and the Band I from the B-ring cinnamoyl grouping[45] (XII).



(XII)



(XIII)

If hydroxyl or methoxyl groups are present in ring-A, resonance contribution of this ring is increased and a bathochromic shift of Band II (approx. 20nm) is observed. Similarly resonance contribution of ring-B is increased due to hydroxyl groups and a bathochromic shift of Band I is produced. The locations of different groups specially hydroxyls on flavonoid nucleus can be easily detected by observing shifts[46] in the UV spectrum by the use of

different reagents. The hydroxyls at different positions ionise with these reagents[47] under different pH, thereby causing shifts in either Band I or II or both.

^{1}H NMR spectra

PMR in flavonoids normally occurs between 0 and 9 ppm. Chemical shift values quoted in parts per million (ppm) on the δ scale. The solvents used for this spectroscopy are Cl_4, $CDCl_3$ and D_2O and their mixtures but DMSO-d_6 is most useful and frequently used.

A - ring protons

In case of 5,7-*di*hydroxyflavone, protons at C-6 and C-8 appear separately as doublets (d, J=2.5 Hz) in range 5.7 - 6.9 ppm. The H-6 doublet consistantly occurs at higher field than the H-8 and glucosylation of 7-OH group causes both signals to shift down the field. The H-6 and H-8 signals are also distinguishable from each other by their widely different paramagnetic induced shifts. C-5 protons in 7-hydroxyflavones is strongly deshielded by the 4-keto group and appears near 8.0 ppm as a doublet (d, J=9 Hz) due to ortho coupling with H-6.

B - ring protons

In case of 4'-oxygenated flavones protons at C-2', 3', 5' and 6' due to free rotation of the B-ring appears as two pairs of ortho coupled doublets (d, J = 8.5 Hz) in the range 6.5 - 7.9 ppm. The H-3', 5' doublet always occurs upfield from the H-2', 6' doublets due to the shielding effect of oxygen substituent and to the deshielding influence of C-ring

functions on H-2' and 6'. The position of the H-2', 6' doublet is dependent upon the level of oxidation of the C-ring.

In case of 3', 4'-dioxygenated flavones, the C-5' proton appears as a doublet (d, J = 8.5 Hz) in the region 6.7 - 7.1 ppm. The signals of C-2' (d, J = 2.5 Hz) and C-6' (q, J = 2.5 and 8.5 Hz) protons usually occur between 7.2 and 7.9 ppm. From the relative chemical shifts of the C-2' and C-6' protons, methylation of the 3'-hydroxyl can be distinguished from that at 4'-hydroxyl.

C - ring protons

The C-3 proton in flavones usually appear as a sharp singlet near 6.3 ppm. As such, it can be confused with C-6 or C-8 proton signals in 5,6,7 and 5,7,8 or 5,6,7,8-oxygenated flavones. In 8-methoxy flavones long range coupling of H-6 with 8-methoxy protons causes the H-6 signals to be slightly broadened and hence the lower intensity.

^{13}C NMR spectra

The carbon resonance of flavonoids appear between 40-200 ppm in ^{13}C NMR and may be conveniently divided into four regions.

(a) 40-85 ppm : C-2 and C-3 resonance of flavonones[48], isoflavonones[49], flavonols[50] as well as methoxy carbon.

(b) 90-110 ppm : C-6, C-8 and two unsubstituted carbons of trisubstituted ring-B in flavones, isoflavonones, as well as C-3 of flavone.

(c) 110-140 ppm : Carbons of mono or disubstituted ring B.

(d) 135-200 ppm : Oxyaryl carbon, olefinic C-O (135 - 168 ppm) and carbonyl carbons (168-200 ppm).

In alkyl substituted flavonoids the signals of carbons of alkyl chain generally appear below 40 ppm.

Functions of flavones [51]

1. Industrial functions

Flavones are prototypes of phenolic polymers which are present in relatively large amounts in some plant species and are of economic importance in leather tanning oil well drilling, adhesives and other industries.

Flavonoids inhibit sulphite pulping and they have been shown to be fungistatic. That is why these are of importance in determing the durability of timber.

2. Physiological functions [52]

Flavones in animals have shown some interesting results such as vitamin - P activity[53]. Chemical uses[54] of rutin for the treatment of cold and injury in conjunction with ascorbic acid and for relieving nutritional stresses[55]. Flavonoids[56] such as Myricetin, Morin. Kaempferol and flavonol glycosides like Quercetin, Rutin as well as flavone Luteolin[57] have been found to possess diuretic action. Quercetin is reported to enhance the nutritive value of butter. Gossypetin and 3, 3', 4'-trihydroxyflavone[58] have been used for their

marked physiological actions. Further, Rhamnetin[59], Quercetin[60] and Quercetrin[61] have shown significant antibacterial activities. Furoflavones isolated from *Pongamia* species have been found to be useful for treatment of certain skin diseases like leucoderma.[62] For example Karanjin exerts appreciable curative effects on skin diseases and is free of irritating and inflammatory effects as observed in case of some coumarin compounds.

3. Flavones as antioxidants

Several flavonoids serve as antioxidants for ascorbic acid, apparently by chelating metals from the reaction mixture. This chelation is dependent primarily on the 3-hydroxy-4-carbonyl and the 3',4'-dihydroxyl groupings (Clements and Anderson, 1961; Samorodova - Bianka, 1965)[63,64]. Ascorbate may, in turn, protect flavonoids from oxidation. Robinetin and Gossypetin have been found to be antioxidants of great potency in case of fats and oils.

4. Flavones as enzyme inhibitors

Flavones and flavonols with 3'-, 4'- and 7-hydroxyl are potent inhibitors of bovine pancreatic ribonuclease (Mori and Noguchi, 1970)[65]. Methoxylation at the 6-or 8-position decreases inhibitory activity. The aglycones were much more effective inhibitors than their glycosides.

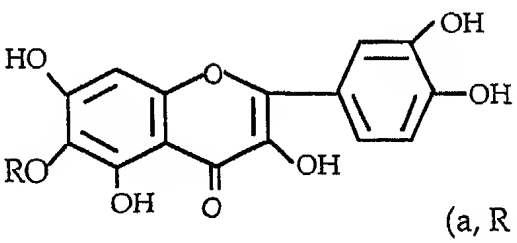
5. Flavones as precursors of toxic substances

Symbiotic nitrogen fixation is an alternative of relying completely on nitrogen fixation by free living bacteria and it is

interesting that exogenously applied quercetin will increase the number of nodules containing nitrogen fixing bacteria in the root system of *Lotus corniculatus* (Molina and Alexander, 1967)[66].

6. Flavones and visible colour

Flavones and flavonols absorb significantly in the range from 330-350 where NAD and NADP cofactors absorb strongly. Patuletin (a) and quercetagetin (b) have been identified as the UV absorbing and reflecting compounds in the nectar guides of Rudbeckia hirta (Thompson *et al.*, 1972)[67].



(a, R = Me)
(b, R = H)

Bloom and Vickery (1973)[68] found that pattern partitioning in yellow flowered *Mimulus luteus* was due to mixture of carotenoids and flavonoids. The carotenoids were uniformly present, while cyanidin-3-glucoside and quercetin-3-glucoside localised within the red spots.

7. Flavones as light screens

These are quite stable to visible and ultraviolet regions of the spectrum. They may play subtle roles as light filters in plant growth. The flavonoids of the primary leaves create a transverse light gradient throughout the coleoptile and shadow the photoreceptor(s) for phototropism in an ideal manner.

8. Photosensitizing and energy transferring compounds

The accumulation of flavonoid aglycones on the outer layer of cuticles may play a role in the photodecomposition of either natural or synthetic toxins falling on these surfaces. Ivie and Casida (1971)[69] reported that quercetin and related substituted 4-chromones accelerate the photoalteration of chlorinated cyclodiene insecticides under field conditions.

9. In control of plant growth and development

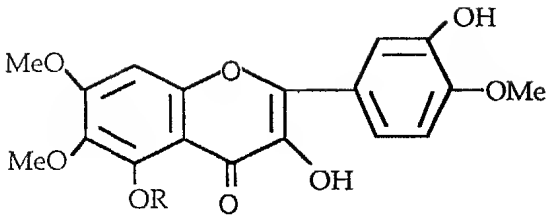
The chemical reactivity of flavonoid - glycosides and their property to complex with proteins ensures that will cause metabolic changes, even if in nonspecific ways, when applied to plants. They may also interact more specifically as cofactors for enzyme reactions requiring hydroxylated phenols as electron acceptors.

10. Antibiotic effects

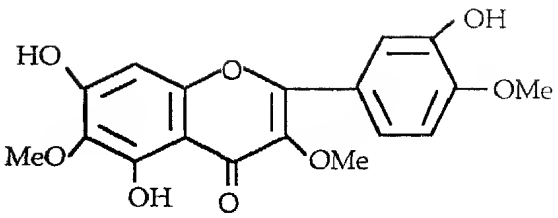
When quercetin was added to cultures of several viruses associated with human maladies, viruses with an envelope were inhibited while those lacking such envelope were moderately or completely, resistant (Puszatai et al., 1966)[70].

11. Flavonoids and malignancy

Several flavonoids are moderately effective against laboratory cultures of malignant cells. Eupatin (c) and eupatoretin (d) (Kupchan et al., 1969)[71] and either centaureidin (e) or 6-demethoxy centaureidin (Kupchan and Bauerschmidt, 1971)[72] are all moderately effective against a carcinoma from the nasopharynx.



(c, R = H)
(d, R = Me)



(e)

12. Toxic flavonoids

There are apparently no reports of flavonoids toxicity in man. Flavones and isoflavones are considered by Anjanexulu and Ramachandrarow (1964)[73] to be inherently toxic to fish, and Chari and Seshadri (1948)[74] found that chrysin (5,7-dihydroxy) and galangin (3, 5, 7- trihydroxyflavone) are considerable toxic to fish while flavones having more hydroxyl groups are only slightly toxic. Methylation of a hydroxyl group increases the toxicity, presumably through increasing lipid solubility.

REVIEW ON TRITERPENOIDS

The term 'triterpenoids' refers to a group of natural products containing thirty carbon atoms based on six isoprene units. Since several substances which contain more or less than thirty carbon atoms and also those which do not strictly follow the isoprene rule, have been isolated and characterised as triterpenoids during the recent years. The invention of highly sophisticated instrumental techniques and the contemporary developments in the biogenetic theories have been mainly responsible for the isolation and identification of new unconventional type of triterpenoids.

Triterpenoids are mainly found in plants but others are elaborated by microbes and as sex pheromones by insects. In nature, we have noticed that several parts of the plants have a particular pleasant smell. This smell is due to the presence of certain volatile oils known as essential oils. Among the chief constituent of essential oils are terpenoids upto C_{15} and their oxygenated derivatives. Triterpenoids are of great biological interest due to many biological activities viz. insecticidal, anthelmintic, antiseptic activity etc. Due to these biological activities they are used in the field of pharmacy and due to their characteristic smell, they are of particular importance in perfumery. Infact, with the advent of the powerful analytical methods and their subsequent development over the years, the structure elucidation of a natural product has become, it may be said, a routine affair. As a consequence, increasing numbers of new

triterpenoids including very complex ones are being isolated and their structures established.

The terpenoids are classified on the basis of number of isoprene units present.

(1)	Hemiterpene	C_5H_8	one isoprene units
(2)	Monoterpene	$C_{10}H_{16}$	two isoprene units
(3)	Sesquiterpene	$C_{15}H_{24}$	three isoprene units
(4)	Diterpene	$C_{20}H_{32}$	four isoprene units
(5)	Sesterpene	$C_{25}H_{40}$	five isoprene units
(6)	Triterpene	$C_{30}H_{48}$	six isoprene units
(7)	Tetraterpene	$C_{40}H_{64}$	eight isoprene units
(8)	Polyterpene	$(C_5H_8)_n$	more than ten isoprene units

Earlier surveys in this field have been made by Holtzem *et al.*,[75] White[76] and Halsall *et al.*[77]. Recently the triterpenoid saponins and their sapogenins have been reviewed by Basu *et al.*[78] and Dalozes[79]. The leading review published by Mahato and Das[80] covers the literature upto 1989.

Recent advances

Occurrence and isolation

Hosenkol-A, reported by Shoji *et al.*[81], is the first example of the natural baceharone triterpenoid of the missing intermediate toshionane and lupane, lanosterol and 24-methylene-lanost-8-en-3-β

–ol, which was generally of animal origin, have been identified in the leaves of *Symphoricarpus allbus.*[82] β–amyrin which is commonly found in plants has been isolated from the fungus *Aspergillus nidulans.*[83] There has been increasing interest in the recent years in triterpenoid hydrocarbons that occur in fossil fuels, sediments and petroleum as they can be used as biological markers[84,85].

Among the recent reports on newer separation methods, the application of droplet - counter- current chromatography (D.C.C.C.) to the separation of mixture of triterpenes, reported by Hostettmann *et al.*[86] deserves special mention.

Structure activity correlation

Relationship between chemical structure and homolytic action and also surface activity of five oleanolic acid glucoside (1; Chart I) have been established[87].

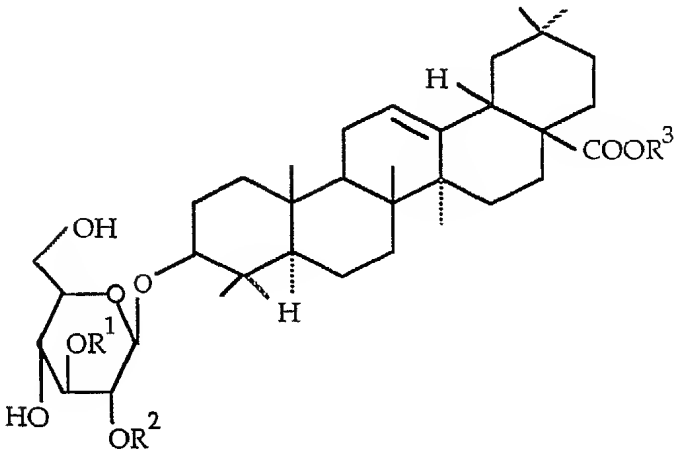
It was shown that monodesmosidic saponins were more homolytic than bidesmosidic ones. The homolytic activity of monodesmosidic side chain decreases with the length and the branching of glucoside chain, surface activity has been found to be major in bisdesmosides and it increases with the length of glucoside chain.

Anticancer activity of nine triterpenoid derivatives on human cancer cells were reported by Ling *et al.*[88] Among the pentacyclic triterpenoids epi-maniladiol (2; chart I) was cytotoxic at 100 μg/ml. The effect was more potent than that of mytomicin C. The presence of a 16-α and a free 3-OH has been found to be essential for cytotoxicity.

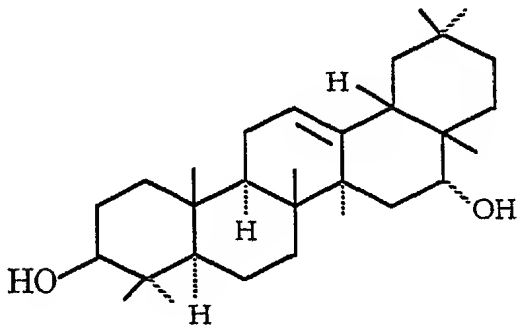
Structure activity studies of some anti-inflammatory oleanane triterpenoid glycosides and related compounds (obtained from the leaves of *Tetrapanox papyriferum*) reported by Ogihara *et al.* [89] deserve special mention here as they have identified the conformational requirement for anti-inflammatory activity in such compounds.

Two of the compounds tested, viz., papyriogenin A and C (3 and 4; Chart I) manifested in these tests almost the same potency as prednisolene. The structure activity - correlations were explored by taking into account the variation in the oxygen function and the molecular conformation. It was found that anti-inflammatory activity of these compounds was favoured when the molecules tended to take a planer conformation.

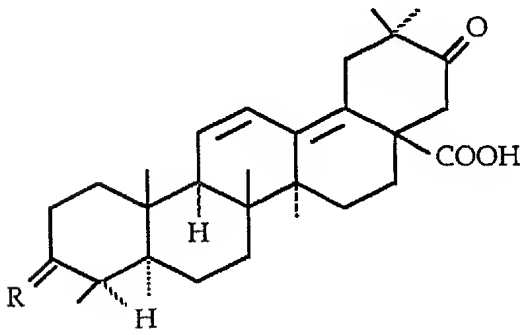
CHART -I, BIOACTIVE OLEAN -12-ENE DERIVATIVES



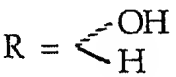
1. R^1 or R^2 = H or *β-D*-glucopyranosyl
   R^3 = H or $\alpha - D$ glucopyranosyl



2



3. R = O

4. R = 

Physical techniques used for structure elucidation of triterpenoids

Infrared spectroscopy (IR)

IR spectroscopy is an important technique which is frequently used for the structure determination of triterpenes. IR spectroscopy has been used as an important tool for the identification of functional groups -OH, O-acetyl, carboxyl, carbonyl with conjugated unsaturation present in different classes of triterpenes.[90-96] Tschesche *et al.*[97] have utilized IR spectroscopy to differentiate ursane and oleanane series by taking the spectra triterpenic acid and their derivative in pyridine. Differentiable spectra were observed in two characteristic region :

(a) Region A - have bands between 1392 - 1335 cm^{-1}

(b) Region B - have bands between 1330 - 1245 cm^{-1}

Number of oleanane series have got two bands in 1392 - 1379 cm^{-1} and 1370 - 1335 cm^{-1} in the region A and three bands of increasing intensity at 1330-1315 cm^{-1}; 1306 - 1299 cm-1 and 1269 - 1250 cm^{-1}in region B. On the other hand the member of ursane series have three bands in both of the two regions in between 1392 - 1386 cm^{-1}, 1383 - 1370 cm^{-1}, 1364 - 1359 cm^{-1} in A and the increasing intensity in the region 1312 - 1308 cm^{-1}, 1276 - 1270 cm^{-1} and 1250 - 1245 cm^{-1}

Tetracyclic triterpenic acids or their esters show only two strong absorptions in these two regions. Acids which do not belong to the type mentioned above possess their intensive bands outside the

described limit. In the case of neutral triterpenes, the characteristic absorption in the B– region have been found missing and the assignments are based only on the absorption in region A for differentiating α–amyrin from β-amyrin and lupeol. IR spectroscopy has been used for differentiating primary, secondary and tertiary axial and equatorial groups[98]. It has been also found to be helpful in determining the presence or absence of hydrogen bonding in triterpene[98].

^{1}H NMR spectra

^{1}H NMR spectroscopic technique is frequently used for the structure elucidation of triterpene sapogenins[99-107].

In the ^{1}H NMR spectra of pentacyclic triterpenes sharp absorptions are found due to methyl esters and acetoxyl groups well defined absorption due to the presence of angular methyl groups are seen in the δ 0.82 - 1.13 ppm region. Certain other functional groups such as olefinic protons have low and diffused absorptions in the region δ 5.66 - 5.44 ppm, δ 3.8 - 4.52 ppm, respectively. Even so their absorptions are helpful for determining certain structural features of the triterpenes[108,109].

^{1}H NMR spectra of series of pentacyclic triterpene were studied and some important correlations between the spectra and their structures were made by Shamma[110]. It was noted that the chemical shift of highest (most shielded) methyl group is partially indicative of the position of carbomethoxy group in triterpenes of ursane or

oleanane series, when carbomethoxy group is present at C-28 position. The absorption peaks appear upfield from δ 0.775 ppm. The proton of the normal trisubstituted double bond in the α– and β -amyrin series absorbs in the region between δ 4.93 and 5.93 ppm. If the double bond is conjugated with the carbonyl function at C-11, the vinylic proton is found to absorb at low field at δ 5.55 ppm and well defined absorption vinyl group appears from δ 0.625 to 1.5 ppm. Acetoxyl protons appear between δ 1.82 – 2.09 ppm^{-} . Other protons present at position C-3, C-12, C-15, C-16, C-22, C-28 appears in the range of δ 3.75 to 5.62 ppm. The shifting of the peak in downfield region indicates the presence of some electronegative group surrounding it. ^{1}H NMR has also been used for the study of conformational equilibrium at varying temperatures. The ^{1}H NMR spectra of acetic acid[111] and polygallic acid[112] assisted in the assignment of the conformation of ring and configuration of hydroxyl group.

^{13}C NMR spectra

^{13}C NMR spectroscopy has solved many problems of structure elucidation and biosynthesis of triterpenoids[113,116]. Identification of saponins which consist of the acid unstable dammarane - type sapogenin has been done excellently with the help of ^{13}C NMR[117]. The ^{13}C FT NMR signals of olean-12-ene in $CDCl_3$ have been reported[118]. The C-16 hydroxylation effect on ^{13}C chemical shift reflect the difference in D-ring conformation. These results have been found useful in structural studies of all triterpenoids.

The ^{13}C signals of oleanane type triterpenes were assigned using known chemical shift rule[119], such as hydroxyl substituent shift acetylation shifts[120], steric[119-121], γ and δ effects[122] and by chemical shift comparison with previously reported data[113-116].

Mass spectroscopy

A detailed study of the mass spectral fragmentation patterns of pentacyclic triterpenes by noting peak shift in various derivatives has been described[123,126]. This has led to important generalization, particularly in Δ^{12} compounds and has been extremely useful in structural elucidation studies of Δ^{12} - oleanane and Δ^{12} - ursane derivative[124,126]. The mass spectrum of Δ^{12} - oleanane and Δ^{12} - ursane are very similar, giving rise to fragments at identical m/z values. The only difference is that in mass spectrum of Δ^{12} - oleanane, the fragmentation at m/z 203 is more intense than the peak at m/z 191 while reverse is true in mass of Δ^{12} -ursane.

Peak M-153

This peak represents the most abundant fragment ion above m/z 218. The mechanism involves homolytic cleavage of 9-10 bond in the molecular ion (a) to afford (b), followed by hydrogen transfer from C-26 to C-7 with concomitant homolysis of the 7-8 bond to afford resonance stabilized species (c) m/z 257 (Chart II).

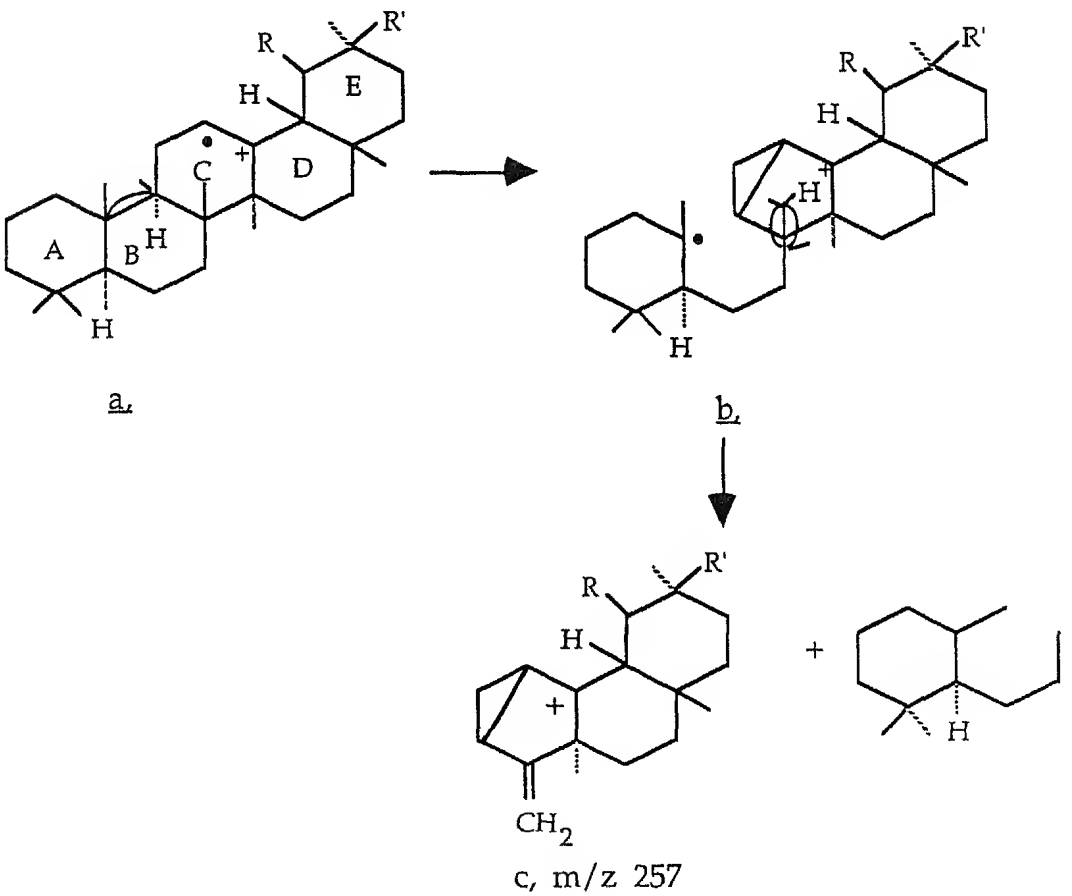
Peak M-192

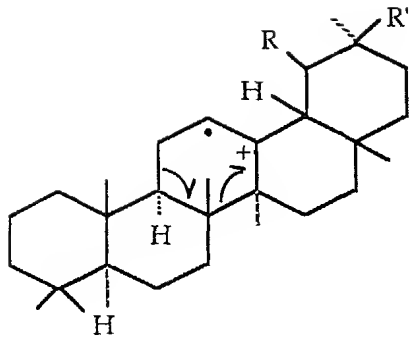
The intense and diagnostically important m/z peak (M-192) has been discussed in considerable detail[123,125,126]. The ion at m/z 218 is formed by retro-Diels-Alder decomposition (a→ d).

Peak M-207

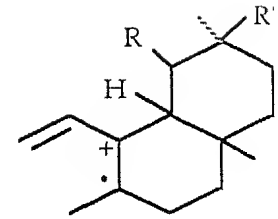
The fragment ion at m/z 203 in the mass spectra of Δ^{12} -oleanane and Δ^{12} -ursane proved to be more interesting peak from a mechanistic stand point. This moiety resulted from further loss of 15 mass units from the retro-Diels-Alder fragment (d). It was found that m/z 203 ion resulted from the loss of the methyl substituents at C-17 or C-20 from (d) (d→f and d→g) (chart II).

CHART - II, MASS FRAGMENTATION PATTERN OF Δ^{12} OLEANANES



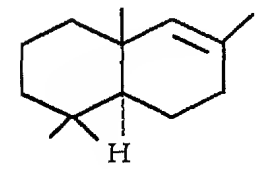


a.



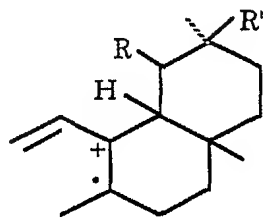
d, m/z 218

+

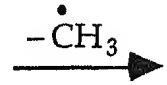


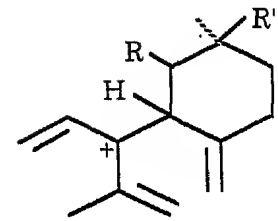
e.

R = CH_3; R' = H or R = H; R' = CH_3

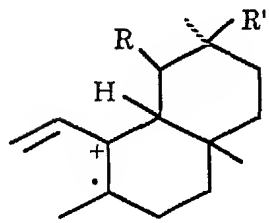


d.

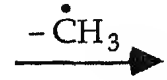


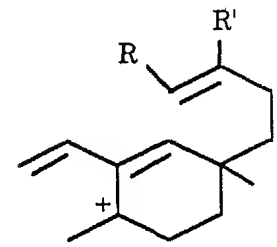


f, m/z 203



d.





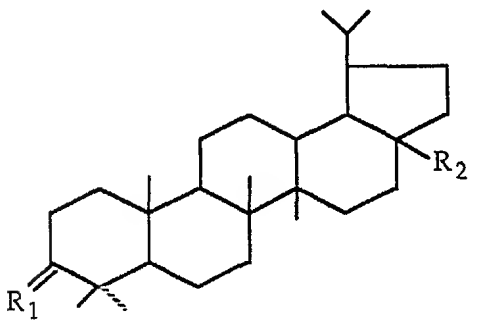
g, m/z 203

Lupane derivatives

This series is characterised by contraction of ring E to a five membered ring to which an isopropyl or isopropenyl group is attached. The loss of 43 mass units C_3H_7 is very pronounced in certain members , but becomes minimal in highly substituted derivatives or in the presence of an isopropenyl function.

a) Saturated lupanes

Lupane-3-one (IIa) exhibits loss of methyl (m/z 411) and isopropyl (m/z 383) groups. The most abundant fragment occurs at m/z 205 corresponding to species hopane, observed to some extent in the spectra of all the pentacyclic triterpenes. A peak at m/z 191 and m/z 189 is also observed in lupane-3-one[126].



II (a) $R_1 = O$, $R_2 = CH_3$

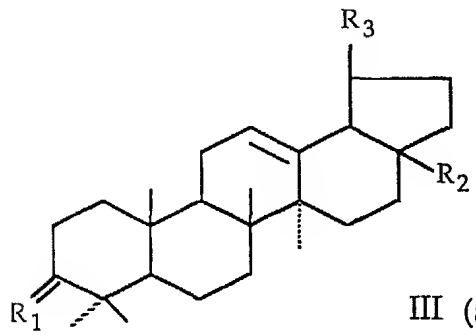
(b) $R_1 = H_2$, $R_2 = CH_3$

(c) $R_1 = H$ (O) Ac, $R_2 = CH_2OAc$

b) Δ^{12} Lupanes

Members of this series (III) exhibit mass fragments at m/z 216 due to retro-Diels Alder decomposition of ring C. The most

characteristic peaks occur at m/z 187, 189, 201 in both 12,13-dihydrolupenone (IIIa) and the corresponding 3-acetate (IIIb) while in 12,13-dihydro-20,30-dihydrolupenol (IIIc) peaks at m/z 189, 191 and 204 are observed.



III (a) $R_1 = O, R_2 = Me, R_3 = C_3H_5$

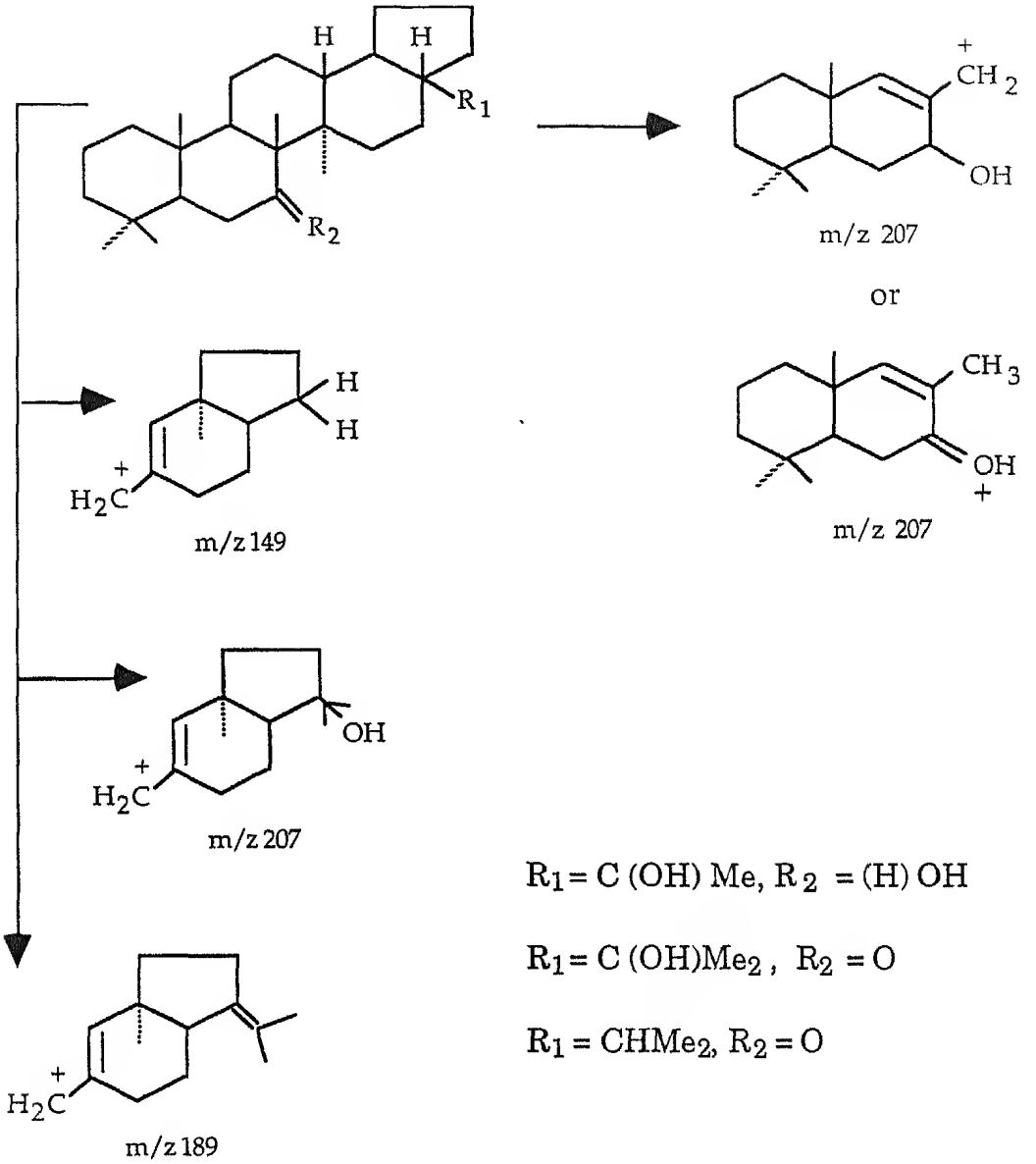
(b) $R_1 = (H)\ OAc, R_2 = Me, R_3 = C_3H_5$

(c) $R_1 = (H)OH, R_2 = Me, R_3 = C_3H_5$

Hopane derivatives

The mass spectral fragmentation pattern of hopane triterpenes and their various derivatives has been described by R.E. Corbett *et al.*[127,128], I. Yosioka *et al.*[129] and J. Schmidt *et al.*[130] The main ion reactions of hopane included two types of cleavage through ring C, (Chart III).

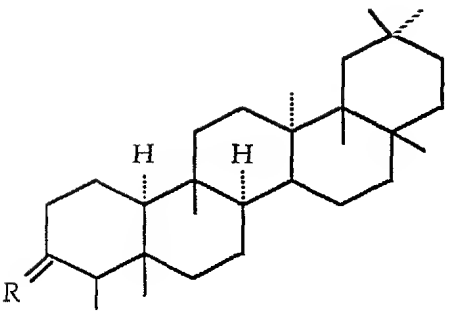
CHART - III



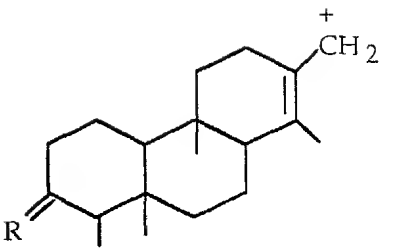
Friedelane derivatives

Friedelane (IV) substituted only in ring A or E gives a characteristic ion of m/z 273 (if R = O). Two additional ions (m/z 302 and 341) are observed in the case of Friedelan-3-one.[123] (see chart IV).

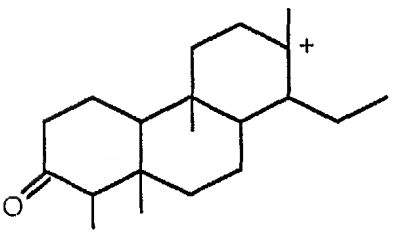
CHART - IV



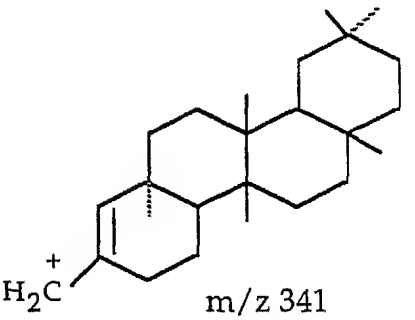
IV (a) R = O
(b) R = H_2



m/z 273, R = O



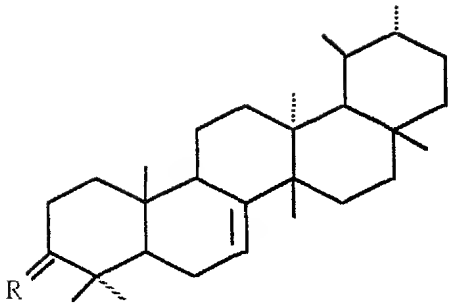
m/z 302



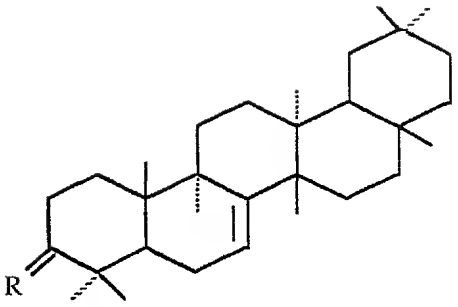
m/z 341

Bauerene and related triterpenes

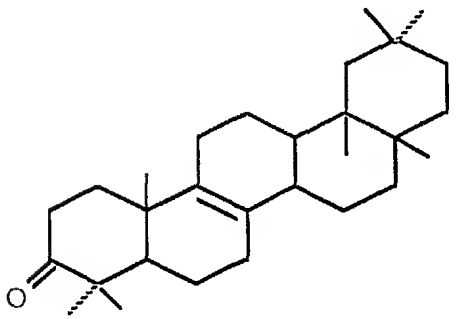
The representatives of this class possess methyl groups at positions 13 and 14, the presence of which characteristically influences the breakdown pattern, the position of double bond (Δ^7, Δ^8, Δ^9), changing only the relative abundance of certain fragments. The triterpenes considered here are baurene (Va) and its 3 ketone (Vb), multiflorene (VIa) and its 3 ketone (VIb), isomultiflorene (VII) and several derivatives of arborinol for which structure (VIII) has been suggested.[123] The increase in steric hindrance in the compound fosters cleavage in ring D and E.



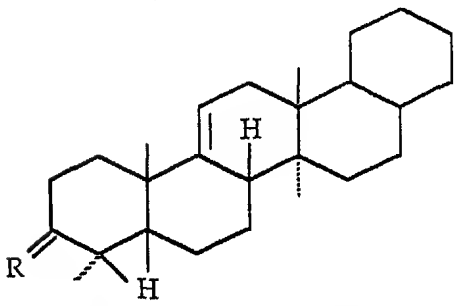
V (a) R = H_2
R = O



VI (a) R = H_2
(b) R = O



VII



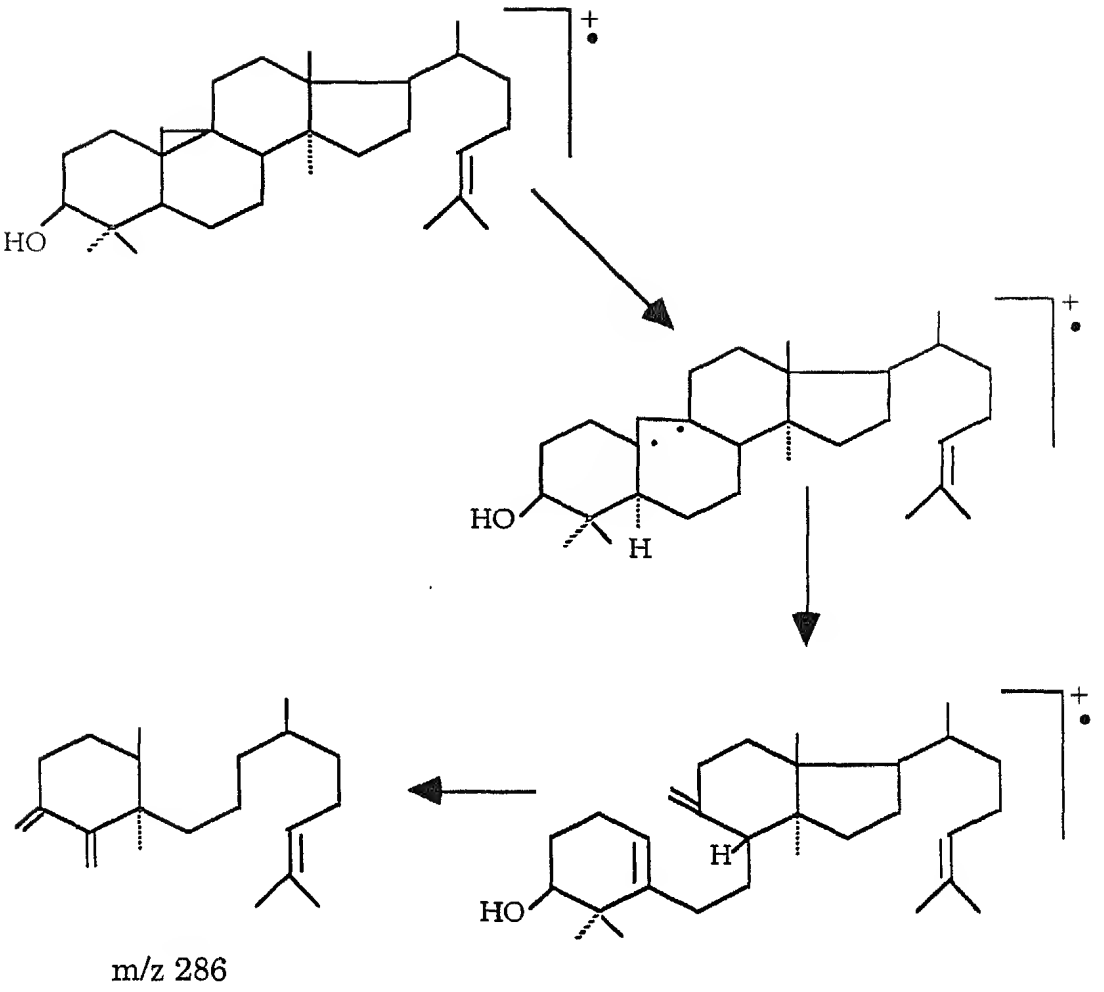
VIII (a) R = (H) (OH)
(b) R = H_2
(c) R = O

Tetracyclic triterpenes

The mass spectral fragmentation pattern of tetracyclic triterpenes and their various derivatives has been described by Audier *et al.*[131,132] Ourisson *et al.*[133], and Aplin *et al.*[134] These authors have demonstrated two distinct types of fragmentation in the mass spectra of lanostane group of triterpene. The two types being –

(i) The formation of ion fragments induced by the presence of 9.19 cyclopropane ring.

(ii) The fragments produced by the cleavage of the side chain.



Biological activities of triterpenoids

The wide occurrence in nature and structural diversity of triterpenoids have evoked considerable interest in their biological activity.

1. **Cytotoxic activity**

A new and highly cytotoxic meliacin type of triterpene isolated from *Aphanamixis grandifolia* is of special interest. A chloroform soluble fraction of the ethanol extract of the leaves of *Bursera klugu*

showed activity against the P-388 lymphocytic leucomia and human epidermoid carcinoma of the nasopharynx.

35-dammarane type triterpenoids and betulin showed cytotoxic effect on developing sea urchins embryos. Depending on their structure and concentration the glycosides caused anomalies in embryo development, arrest of egg division and blastomerelysis. The triterpenoids pachymic acid, tumulosic acid and their 7,9(11)-dihydro derivatives obtained from *Poria cocos* possess cytotoxic activity in vivo against hepatoma. Panaxoside a aglycone and panaxodiol diglucoside had the highest cytotoxic activity.

2. Cytostatic activity

Iguesterin and their related compounds isolated from the root bark by *Maytenus canariensis* showed powerful cytostatic activity against He La cells in vitro though no conclusive relationship was established between the activity and structure.

3. Antimicrobial activity

The pentacyclic triterpene glycosides of oleanic acid and hederagenin with a free carboxylic group at C-28 or C-27 possess the highest fungicidal activity. A number of tetranortriterpenoids isolated from plants of Meliaceae family have been found to possess antimicrobial properties. Toonacilin and 6-acetoxytoonacitin, isolated from *Toona ahata*, showed antifeeding activity against *Epilachna vervestis*. The root bark of the East African tree *Trichilia roka* yielded a series of new limonoids, the trichilins which exhibited a

broad spectrum of insect antifeedant and pesticidal activity against the Southern army worm, Mexican bean beetle, Tobacco horn worm and other pest insects.

4. Herbicidal activity

Two compounds with the cucurbitane skeleton, cucurbitacins D and I isolated from seeds of *Purshisa tridenta* showed the seed germination inhibitory property and a large number of naturally occurring compounds with the lanostane skeleton from, *Neamatoloma fasciculare* and *N. membranacea* also showed the growth inhibitory properties on young plants.

5. Anti-inflammatory activity

Reduced forms of glycyrrhetinic acid showed marked antiallergic and antiulcer activities in mice. Moreover, carbenoxolone, the succinic acid derivative of glycyrrhetinic acid, also possesses the antiulcer activity.

6. Effect on metabolism

The inhibitory activity of carbenoxolone on the prostaglandin metabolizing enzymes, 15-hydroxy-PG dehydrogenase and Δ^{13}-PG reductase has been studied in vivo. In another study the counteraction of carbenoxolone with the stimulation of lypolysis induced by glucagon in chicken adipose tissue in vitro was observed.

The effect of three triterpenic acids and sporidesmin on enzyme activities of rat liver plasma has been studied. Laterogenin

and 22B-angeloyloxy oleanic acid partially inhibited both Mg^{++} ATPase and Na^{+}-ATPase at low concentration whereas asiatic acid was found to be an effective inhibitor of Na^{+} K^{+}-ATPase at low concentration but did not inhibit Mg^{2+} ATPase.

7. Effect on biosynthesis

Fusidic acid possess not only antimicrobial activity but also exhibits a unique in vivo stimulation of labelled amino acid incorporation into proteins of liver, kidney, brain and muscles of both male and female rats. Hederagenin, a pentacyclic triterpenoid had a marked inhibitory effect on the rate of biosynthesis in rat marrow.

AN IMPORTANT TOOL FOR THE STRUCTURE ELUCIDATION OF THE GLYCONE PART : PHYSICAL TECHNIQUES :

^{1}H NMR

^{1}H NMR clearly establish the nature *(α,β)* of linkage at the glycosidic point of the saponins. The anomeric protons of various sugars show peak in the region between δ 4.00 - 6.30 ppm. D-sugars generally have β – linkages with high coupling constants (J = 6-9 Hz) whereas rarely occurring α– linkages of D-monosaccharides have low values (J = 2-9 Hz). The coupling constants of the commonly α -linked L – rhamnose (J = 2 Hz) and α- arabinose (J = 6-8 Hz) distinguishes these sugars from each other as well as other sugars.

^{13}C NMR

^{13}C NMR spectra have been found to be useful in the structure elucidation of glycone part of saponins in two ways.

(a) The type of linkages at glycosidic points can be confirmed.

(b) The exact position of linkages in the glycone part is established.

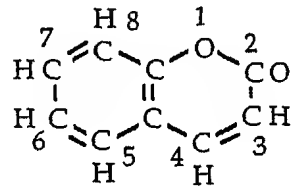
The chemical shift values of some methyl monosaccharides are known[135]. The type of linkages at glycosidic points can be determined by comparing the chemical shift values observed, with the reported values for the methyl pyranoside (s). It has been found that ^{13}C NMR signals shift in the change from aglycone alcohol and pyranose into glycopyranoside, that is glycosidation shift[136].

There are characteristic of chemical and steric environments of the hydroxyl group in which the glycosidation takes place. This discovery has become important and useful for determining the glycosidation position in an aglycone moiety and the kinds and sequence of sugar moiety present in a natural glycoside without chemical degradation as well as for assigning ^{13}C NMR signals of glycoside[137].

REVIEW ON COUMARINS

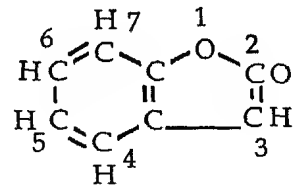
Coumarins are most important members of lactones. Lactones are commonly distributed in nature. The most important members of this class occuring in essential oils are the coumarins and coumarone derivatives, isolated from aromatic plants, identified and synthesized. For our purpose, we may subdivide these compounds into two groups.

1. The coumarins proper and their derivatives, derived from o-hydroxy cinnamic acid or by the fusion of one benzene nucleus with one 1,2-pyrone, also called 1,2-coumarin ring.



Coumarin (I)

2. The coumarones result by the fusion of one benzene nucleus with one furan ring.



Cumarone (II)

Coumarone itself does not occur in essential oils but mainly derivatives of coumarone have been reported.

The isolation of coumarin was first reported by Vogel in Munnich in 1820[138]. The name "coumarin" originated from a Caribbean word "coumarou" for the tonaka tree, known botanically as *Coumarouna odorata* Aubl.

Coumarin, of wide distribution in nature, has been found in more than sixty plants belonging to about twenty four Natural Orders. Most of them are isolated from Umbelliferae, Rutaceae and Leguminosae and a few from animals and micro-organisms. They absorb a wide range of UV light and generate intense fluorescence (usually blue)[139]. Coumarin and its derivatives occur both in the free state and as glycosides.

Naturally occuring coumarins are classified as :

a) Coumarins substituted with one or more hydroxyl and /or methoxyl groups in the benzene ring (e.g. umbelliferone 1).

b) Coumarin substituted with isoprenoid residues (e.g. aurapten 2, suberosin 3, xanthyletin 4, samidin 5) .

c) Furanocoumarin.

   (i) Linear furanocoumarin (e.g. psoralen 6)

   (ii) Angular furanocoumarin (e.g. angelicin 7)

d) 3-phenylcoumarins (eg. pachyrrhizin 8)

e) 4-substituted coumarins

   4-alkylcoumarins (e.g. mammein 9)

4-hydroxycoumarins (e.g. dicoumarol 10)

4-phenylcoumarins (e.g. dalbergin 11).

f) 3-phenyl-4-hydroxycoumarins (eg. scandenin 12)

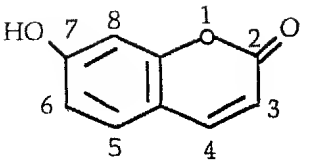
g) 3,4- benzocoumarins (e.g. ellagic acid 13)

The occurrence of coumarin types a, b and c is very frequent while that of the others is rather rare[140] :

Biogenetically coumarins are derived from skikimic acid, but 3-phenyl coumarin derivatives (i.e. type d and f) belong to isoflavonoids and 4-phenyl coumarins to neoflavonoids.

Physiological effects

The physiological effects of coumarins and coumarin derivatives on man and animals are very interesting and hence need attention. On human beings, coumarin has a slightly toxic effects. The first dose to the extent of 4g produces the symptoms of illness and weakness. A dose of approximately 5g kills a sheep, whereas a dose of about 40g is fatal for horses and cattle. The hydroxycoumarin have been found less effective, although toxicity increases considerably on methylation. Pimpinellin, peucedanin and osthruthin possess only little toxicity on mice, rats and guinea pigs. Natural coumarins are highly effective substances on fish. Coumarin is lethal to fish in as low a concentration as 1g in 6800 cc. of water . Some furanocoumarins are also highly toxic to fish.



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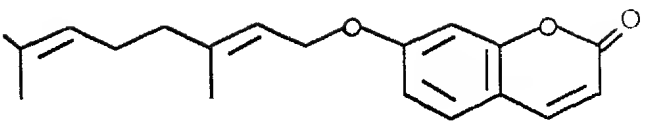

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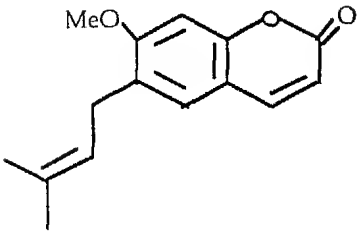

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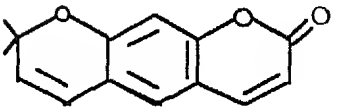

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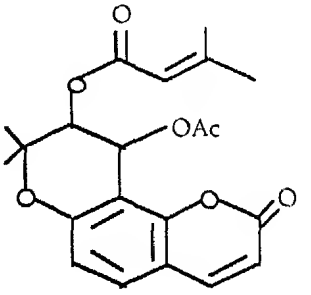

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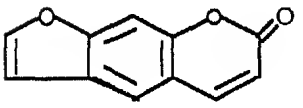

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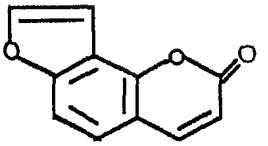

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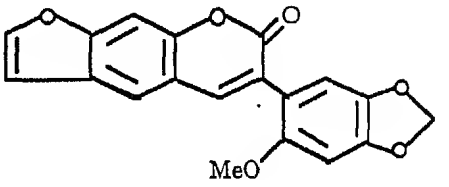

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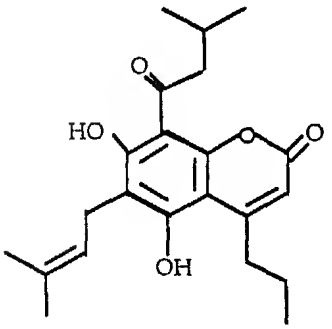

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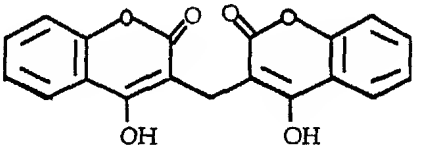

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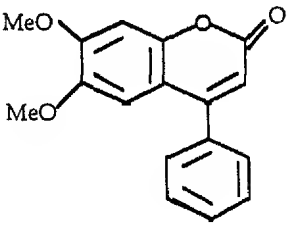

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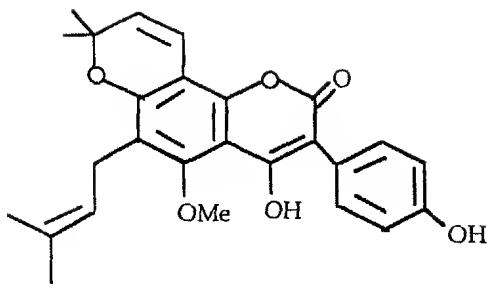

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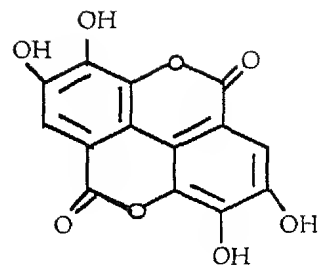

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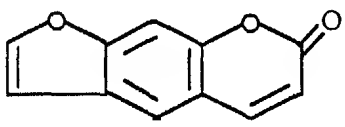
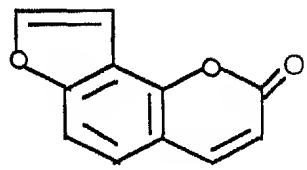
Furanocoumarins

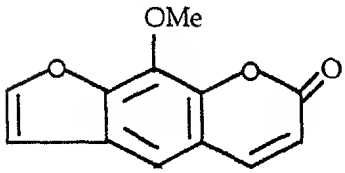
Furanocoumarins occur especially in plants of the families, Umbelliferae and Rutaceae. Many furanocoumarins have also been synthesised. Furanocoumarins have a furan ring fused with 2H-1-benzopyran–2-one (coumarin) nucleus. The parent member of the linear furanocoumarin is psoralen while that of the angular type is angelicin (also called isopsoralen).



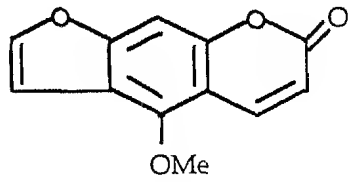
Psoralen



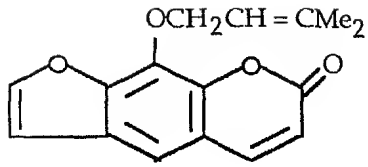
Angelicin



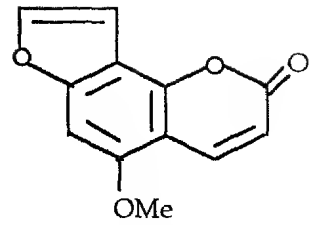
Xanthotoxin



Bergapten



Imperatorin



Isobergapten

Representatives of the linear type are psoralen, bergaptol, bergapten, xanthotoxin, imperatorin, isoimperatorin and isopimpinellin. Representatives of the angular type are angelicin and pimpinelin.

Biological activities of furanocoumarins

Furanocoumarins like other coumarins exhibit a variety of biological activities[141]. However, furanocoumarins are especially noted for their photosensitizing effects, i.e., the biological effects exerted by them upon irradiation with long wavelength UV light.

1. Photosensitizing effects

Some plant extracts and juices increase photosensitivity of skin. Application of these substances on the skin followed by exposure to sunlight causes erythema and pigmentation. Intense exposure may lead to hyperpigmentation and occasionally vesiculation of the skin. The substances responsible for this action on skin are furanocoumarins present in such plants. Dermatitis may be caused by some common plants or plant products such as Cologne water (containing bergamot oil), by rue, parsnip, fig leaves or celery.

The best known photosensitizing effect of the furanocoumarins is erythema of human or guinea pig skin, appearing after the application of the substances on the skin followed by exposure to long wavelength UV light or sunlight. Erythema (edema and vasicle may also occur) appears after a latent period of several hr, lasts a few days and is followed by dark pigmentation[142-145].

A number of furanocoumarins such as xanthotoxin (also called methoxalen, 8-methoxypsoralen), imperatorin (8-isoprenyloxy-psoralen) and 2', 4, 8- trimethoxypsoralen also called trioxalen are used for the treatment of leucoderma which is characteristic of vitiligo. These furanocoumarins are either given orally or applied locally on the leucodermic spots which are then very carefully exposed to sun or long - wavelength UV light. In many cases repigmentation is obtained[146].

Seeds of *Psoralea corylifolia* (Babchi) are used in India in the treatment of leucoderma and other skin diseases. The curative action of *Psoralea* in leucoderma is due to psoralen and isopsoralen (angelicin)[147]. The furanocoumarins, viz., xanthotoxin, bergapten and imperatorin were isolated from the fruits of *Ammi majus* [148,149] are used since ancient times in Egypt for the treatment of leucoderma. Of the three furanocoumarins, xanthotoxin and bergapten are active as dermal photosensitizing agent.

2. Photobiological effects

When irradiated at 365 nm, furanocoumarins may produce some interesting photobiological effects like:

a. **Killing of bacteria**

Only dermal photosensitizing furanocoumarins produce this effect when bacterial cells are irradiated.[150,152]

b. **Formation of mutants**

Mutagenic effect of xanthotoxin were observed on *Escherichia coli*[153] and *Sarcina lutea*. Psoralen also produced mutagenic effect on *Drosophila melanogaster*[154].

c. **Inactivation of tumour cells**

Musajo and coworkers[155] found that Erlich ascites tumour cells lost tumour-transmitting capacity after irradiation in the presence of various furanocoumarins, e.g. psoralen, xanthotoxin, bergapten, 8-methylpsoralen and trioxalen.

d. **Formation of giant cells**

This effect was noticed on irradiation of mammalian cells adapted to *in vitro* growth in the presence of psoralen.[156] The formation of polynuclear cells in sea urchin eggs with sperms irradiated in the presence of psoralen has also been reported.[157]

e. **Inactivation of viruses**

Some DNA viruses were completely inactivated when irradiated in the presence of psoralen while some RNA viruses proved more resistant.[158]

Fluorescence, colours and chromogenic reactions of coumarins

The most obvious physical property of most of the natural coumarins is the fluorescence they show in UV light (365 nm)[159]

7-alkoxy coumarins generally have a purple fluorescence whereas 7-hydroxy coumarins and 5,7-dioxygenated coumarins tend to fluorescence blue. Furanocoumarins generally possess a dull yellow fluorescence. The colours given by furanocoumarins may be intensified by spraying plates with 10% KOH in methanol or 20% antimony chloride in chloroform.[160]

Many 7-oxygenated coumarins even fluorescence in visible light especially if dissolved in concentrated H_2SO_4. An intense green fluorescence in alkaline solution, which can disappear, is indicative of 7-hydroxy coumarin[161].

Chromogenic reactions

1. Phenolic hydroxyl groups are detected by spraying chromatograms with 1% aqueous solution of ferric chloride[162]. Blue or green colour is produced.

2. Epoxide[163] groups are found in many natural coumarins and can be detected at the microgram level due to their susceptibility of cleavage by acids.

3. The Emerson reagent[164], devised for the detection of phenolic groups, was found to be useful for differentiating between linear (psoralens), and angular (angelicins) furanocoumarins. Only the angular one gave red violet colour when paper chromatograms were sprayed with 0.5 M alcoholic KOH and then sprayed with aqueous solutions of aminoantipyrine and potassium hexacyanoferrate (III).

4. The Emerson reagent[164] has been found to give pink colours for the acyloxy dihydro pyranocoumarins selinidin and pteryxin and for the phenol glucosides, magnolioside and daphnin.

Thin layer chromatography

A number of hydroxyl coumarins, dihydroxyl coumarins and coumarin glycosides have been separated on silica gel using toluene : ethyl formate: formic acid (5:4:1) or chloroform : acetic acid : water (4:1:1)[165] as eluent TLC on silica gel with development in hexane : ethyl acetate (3:1) was used to separate bergapten from bergamot oil. The separated coumarins were identified by their R_f values and characteristic fluorescence under UV light.[166]

Spectroscopic methods

UV absorption spectra are useful for distinguishing coumarins from chromones. Chromones normally have a strong absorption at 240-250 nm whereas coumarins usually have a minimum at this wavelength.

Infrared spectroscopy is principally of use in detecting functional groups. Coumarins are isomeric with chromones but the two classes differ considerably in their IR spectra. The carbonyl stretching in coumarins (α–pyrones) is observed in the region 1700-1750 cm^{-1} whereas in chromones (γ-pyrones), it is found at 1650 cm^{-1}.[167]

^{1}H NMR spectroscopy

A wide range has been applied to the structural elucidation of naturally occuring coumarins.

A pair of doublet, (J = 9.5 Hz) centred at δ 6.1 - 6.4 and 7.5 - 8.3 in ^{1}H NMR spectrum is for H-3 and H-4 protons, respectively. The majority of natural coumarins have an oxygen function at C-7 which by electron release leads to an increase in the electron density at C-3 compared to coumarin (I), thereby causing the resonance of H-3 to move to higher field by 0.17 ppm[168]. Electron release by an oxygen substituent at C-5 has a similar, though smaller effect since this involves of less favourable orthoquinonoid electronic distribution.[168]

The H-4 resonance is found in the region δ 7.5 - 7.9 in coumarins lacking a C-5 oxygen function.[169] An oxygen or alkyl substituent at C-5, however, characteristically shifts the resonance of H-4 downfield by ~0.3 ppm (the peri effect), H-4 being found at δ 7.9 – 8.2[170,171].

^{13}C NMR spectroscopy

^{13}C NMR spectrum has proved to be particularly valuable for identifying coumarin glycosides. Not only can the glycosidic moiety be characterized but also the exact position of sugar substitution and the anomeric configuration can be deduced.[172]

The chemical shifts of the carbonyl carbon atom has been

found to be approximately the same, 160 ppm, for most of the coumarins. For coumarin (I), the following chemical shifts (in ppm relative to TMS in $CDCl_3$) have been observed.

Carbon	Chemical shifts (ppm)
C-2	160.4
C-3	116.4
C-4	143.6
C-4a	118.8
C-5	128.1
C-6	124.4
C-7	131.8
C-8	116.4
C-8a	153.9

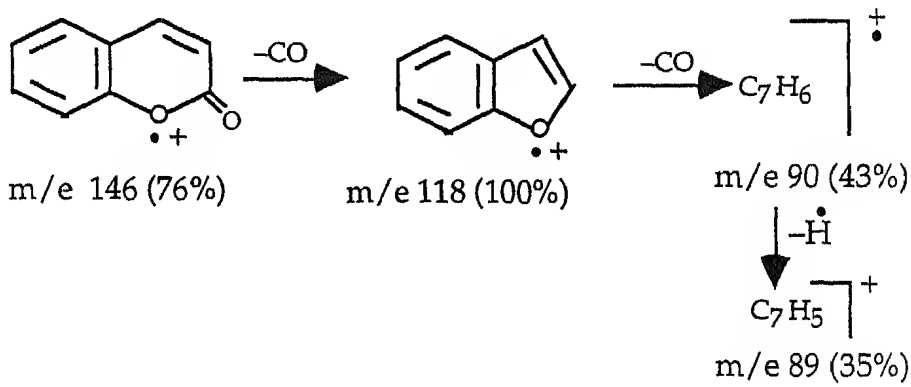
The effect of hydroxyl and methoxyl groups on the benzenoid ring is quite characteristic in that, the signal from the newly formed quaternary carbon atoms is found 30 ppm downfield from the value observed in coumarin (I) while the carbons ortho and para to the substituent move upfield ~ 13 and ~ 8 ppm respectively.[173]

Mass spectroscopy

It has been used for the determination of molecular formula by accurate measurement of the molecular ion.

Coumarin (I) was shown by Barnes and Occolowitz[174] and Vul'fson *et al.*[175] to give a strong molecular ion peak (M^+, m/e 146, 76%) on electron impact and a base peak (m/e 118, 100%), 28 mass units lower[176]. Base peak resulted from the molecular ion by loss of carbon monoxide (CO)[176].

Most chemists use the benzofuran ion to represent the species which results from the loss of CO from the coumarin carbonyl group and which is a characteristic feature of the mass spectra of most coumarins. The benzofuran ion decomposes further by consecutive loss of CO and a hydrogen atom.[176]



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CHAPTER - 2

CHEMICAL EXAMINATION OF THE WHOLE PLANT OF

Cassia occidentalis

The air-dried and finely crushed whole plant of *Cassia occidentalis* was first deffatted by extraction with hexane. The defatted plant was extracted with boiling ethanol. The alcoholic extract was concentrated under reduced pressure and the concentrated alcoholic extract was poured into ice cold water whereby an aqueous solution and a water insoluble residue were obtained. The concentrated aqueous solution was chromatographed over a flash column using different solvents of increasing polarity, viz. hexane, benzene, ethyl acetate and methanol, respectively. Individual fraction was worked out separately. Benzene-ethyl acetate fraction was found to contain two compounds.

The water insoluble fraction was successively extracted with hexane, benzene, ethyl acetate and methanol in a soxhlet extractor.

Single compound has been isolated from benzene : ethyl acetate fraction. All the three compounds were crystallised.

Homogeneity and purity of these compounds were checked by chromatography.

Thus, in three compounds, two flavonoid glycosides and one triterpene were isolated from the whole plant of *Cassia occidentalis*, are identified as:

Compound CO -1 : 3,2'-dihydroxy-7,8,4'-trimethoxyflavone-5-*O*-[-*β*-*D*-glucopyranosyl (1→2)]-*β*-*D*-galactopyranoside.

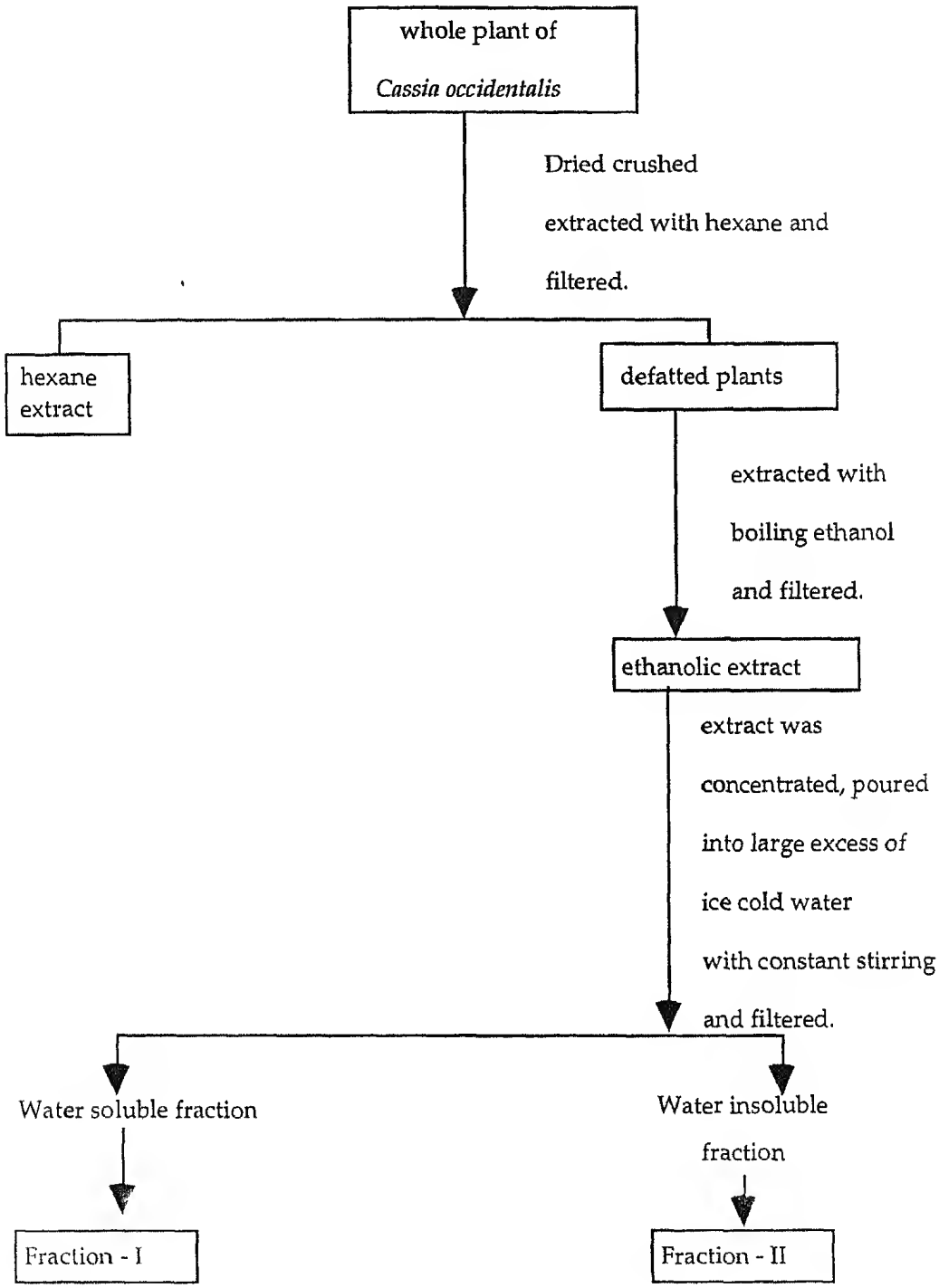
Compound CO-2 : Apigenin-7-*O*-*β*-*D*-allopyranoside

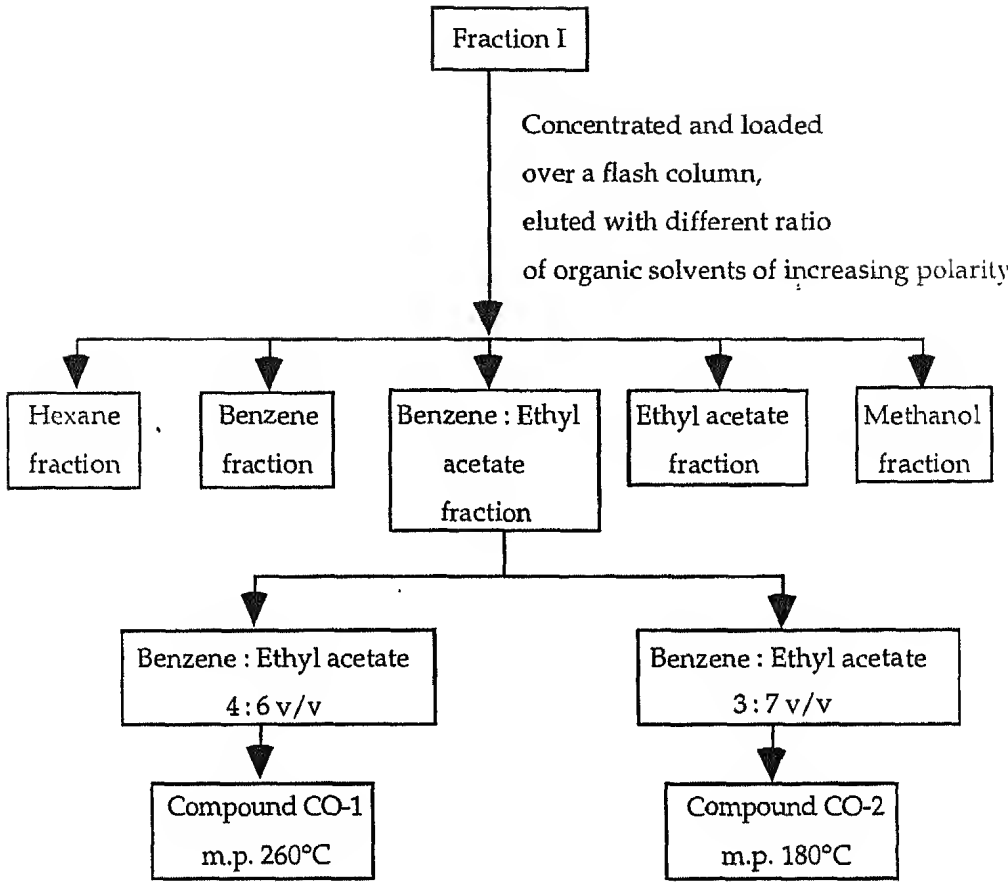
Compound CO-3 : 2*α*,3*β*, 19*α*, 23–tetrahydroxy-urs-12-en-28-oic acid.

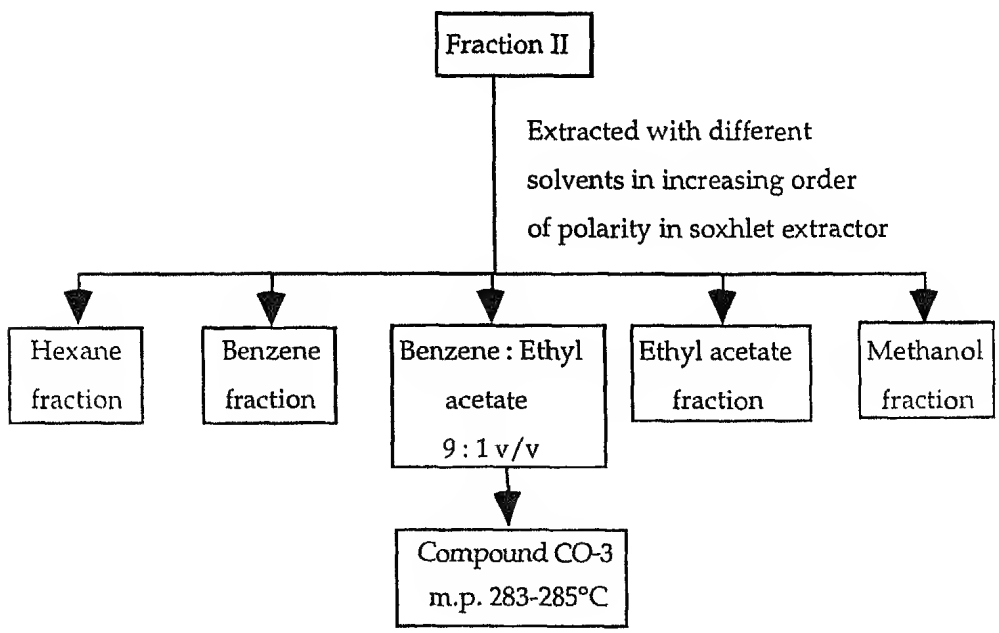
The compounds CO-1 and CO-2 were found to be new and have not been reported earlier from any other plant sources. The compund CO-3 has been reported from other plant sources also.

The detailed study of these compounds, CO-1, CO-2 and CO-3 have been given in this chapter under section I, II and III, respectively.

Scheme of isolation and purification of compounds from the whole plant of *Cassia occidentalis*




Fraction I
Concentrated and loaded
over a flash column,
eluted with different ratio
of organic solvents of increasing polarity
Hexane fraction
Benzene fraction
Benzene : Ethyl acetate fraction
Ethyl acetate fraction
Methanol fraction
Benzene : Ethyl acetate 4 : 6 v/v
Benzene : Ethyl acetate 3 : 7 v/v
Compound CO-1 m.p. 260°C
Compound CO-2 m.p. 180°C


Fraction II
Extracted with different
solvents in increasing order
of polarity in soxhlet extractor
Hexane fraction
Benzene fraction
Benzene : Ethyl acetate 9 : 1 v/v
Ethyl acetate fraction
Methanol fraction
Compound CO-3 m.p. 283-285°C

Experimental

The whole plant of *Cassia occidentalis* was collected from Allahabad, U.P.

All the spectral studies throughout the work have been done using following instruments.

UV spectra was recorded on a Beckman-DK2 spectrophotometer.

^1H NMR spectra of compound CO-1 was recorded at 200 MHz in $CHCl_3$ and ^{13}C NMR spectra at 25 MHz in $CDCl_3$. ^1H NMR spectra of compound CO-2 was recorded at 300MHz in DMSO-d_6 and ^{13}C NMR spectra at 75.46 MHz in DMSO-d_6 by using TMS as internal standard employing the FT mode. ^1H NMR spectra of compound CO-3 was recorded at 90 MHz in $(CD_3)_2CO$ and mass spectra on a Jeol-D 300 instrument.

Extraction with hexane

The air dried and finely crushed whole plant of *Cassia occidentalis* (2.5 kg) was defatted by extraction with boiling hexane (4 x 2.5 litres) under reflux.

Extraction with ethanol

The hexane defatted plant was subsequently extracted exhaustively with ethanol (4 x 2.5) under reflux. The alcoholic extract was concentrated under reduced pressure in a rotary evaporator The concentrated extract (600 ml) was poured into a large excess of ice-cold distilled water with constant stirring whereby a water soluble solution

(fraction I) and a water insoluble residue (fraction II) were obtained which were separated by filtration.

Fraction I

Fraction I was concentrated (300 ml) under reduced pressure. The concentrate was loaded over a flash column and eluted with hexane, benzene, ethyl acetate and methanol, respectively in the order of their increasing polarity.

From hexane, benzene, ethyl acetate and methanol fractions no compound could be isolated. From benzene : ethyl acetate fraction two compounds namely CO-1 and CO-2 were isolated. Benzene : ethyl acetate 4 : 6 v/v and 3 : 7 v/v fractions were concentrated under reduced pressure separately. The concentrate revealed the presence of compounds viz., a flavonoid glycoside CO-1 (350 mg), m.p. 260°C, a flavonoid glycoside CO-2 (300mg), m.p. 180°C. These flavonoid glycosides were crystallised. Purity and homogeneity of individual glycoside was checked by silica gel 'g' plates.

Fraction II

The water insoluble fraction was successively extracted with hexane, benzene, ethyl acetate and methanol in a soxhlet extractor. Each fraction was worked out separately.

From hexane, benzene, ethyl acetate and methanol fractions practically nothing could be obtained. Benzene-ethyl acetate (9 : 1v/v) fraction was concentrated under reduced pressure. The concentrate revealed the presence of one compound viz., triterpene CO-3 (450 mg), m.p. 283-285°C.

SECTION - I

Characterisation of compound CO-1

3,2'-dihydroxy-7,8,4'-trimethoxyflavone-5-*O*- [-β-*D*-glucopyranosyl (1→2)]-β-*D*-galactopyranoside

The chromatographically homogeneous crystalline compound, isolated from the ethanolic extract of *Cassia occidentalis* on the basis of elemental analysis, was found to have molecular formula $C_{30}H_{36}O_{18}$, m.p. 260°C. The presence of sugar moiety was confirmed by positive Molisch's test[1]. The glycoside neither reduced Fehling's solution nor gave purple colour with AHP reagent[2] suggested that the reducing group was not free and involved in glycosidic linkage. On acid hydrolysis it gave an aglycone and two sugars, which were identified as *D*-galactose and *D*-glucose on the basis of co-chromatography with their authentic samples.

Aglycone

The aglycone was analysed for $C_{18}H_{16}O_8$ [M^+360]. The compound gave following colour reactions characteristic of flavones.

1. It gave pink colour with Mg/HCl (Shinoda test)[3].
2. It gave pink colour with sodium amalgam and hydrochloric acid (Asahina *et al.*)[4].
3. An orange red solution with conc sulphuric acid[5].
4. When exposed to vapours of ammonia, a spot of the ethanolic solution of the compound on a filter paper turned yellow[6].
5. It did not give any positive colour with 2,4-dinitrophenyl hydrazine (Horhammer *et al.*)[1].
6. It was not reduced by sodium borohydride, thus confirming its flavone nature.

7. It gave olive green colour with 1% ethanolic ferric chloride solution[8].

8. It gave positive pew's test[9,10].

9. It gave yellow colour with lead acetate.[6]

10. It produced bright yellow colour with zirconium oxychloride in presence of citric acid.[11]

All these chemical reactions suggest that it must be a 3-hydroxy flavone (flavonol).

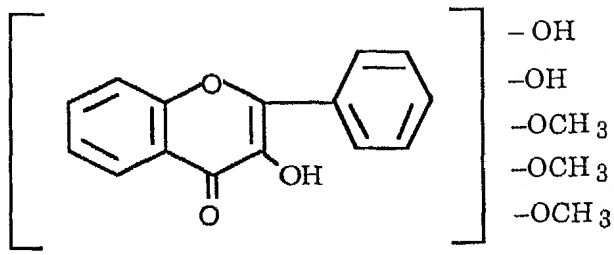
The nature of the compound as a flavonol was further confirmed by its UV spectrum. Two absorption maxima at λ_{max} 360nm (band I) and 255nm (band II) are characteristic of flavonol[12, 13]

^{1}H NMR spectrum of the aglycone showed the presence of four aromatic protons. This means the compound is penta substituted flavonol. The aglycone on acetylation with acetic anhydride and pyridine at room temperature formed triacetate, indicating the presence of three hydroxyl groups. The presence of three hydroxyl groups was further confirmed by ^{13}C NMR which showed signals at δ 109.0, 156.8 and 148.3 ppm. These were assigned for the carbon atom containing –OH group [14].

In ^{1}H NMR spectrum three singlets at δ 3.85, 3.95 and 3.98 ppm each corresponding to three protons, showed the presence of three methoxyl groups in the aglycone. The presence of three methoxyl groups was further confirmed by ^{13}C NMR spectral studies, which

showed quartet signals at δ 56.5, 61.0 and 55.8 ppm[14].

The compound can thus be represented as :



The position of these hydroxyl and methoxyl groups could be assigned on the basis of colour reactions and spectral studies.

The ^{1}H NMR spectrum of the aglycone exhibited four signals at δ 6.50 (1H, s, C-6) , δ 7.10 (1H, d, *J* = 2 cps), δ 7.20 (1H, dd, *J* = 2 & 9 cps) and δ 7.90 (1H, d, *J* = 9 cps) ppm corresponding to four aromatic protons. Coupling constant *(J)* values of these protons indicated that one proton is meta coupled, one proton is both ortho and meta coupled and one proton is ortho coupled. This clearly showed that all the three protons are present in the same ring. Thus, 2',4' disubstituted should be B-ring, because three positions are not available for these three protons in the A-ring[14]. Thus, the position of these protons are at C-3', C-5' and C-6'. The ^{13}C NMR spectrum of the compound showed signal at δ 182.0 ppm for the carbonyl carbon, C-4 of the compound.

Position of -OH and -OCH_3 groups

From above explanation, it has been revealed that C-3, C-5, C-7, C-8, C-2' and C-4' positions are for three hydroxyl and three methoxyl groups.

In UV spectrum there was bathochromic shift in both bands (band I & II) in presence of $AlCl_3$ indicated the presence of two hydroxyl groups at position C-3 and C-5[13,15]. The position of -OH groups at C-3 and C-5 were further confirmed by 60 nm bathochromic shift of band I with addition of $AlCl_3$-HCl[16].

The shifts observed in the UV spectral bands of compound in presence of different reagents have been tabulated below :

Table - I

Solvent	Band II	Band I	Shift (Band II) nm	Shift (Band I) nm
CH_3OH	255	360	-	-
$CH_3OH/AlCl_3$	255	420	-	60
$CH_3OH/AlCl_3/HCl$	255	420	-	60
CH_3OH/fused NaOAc	265	385	10	20
CH_3OH/NaOMe	275	405	20	45

The ^1H NMR spectrum of the aglycone displays signal for one proton singlet at a δ 13.25 due to chelated phenolic hydroxyl group. The ^{13}C NMR spectrum of the compound shows desheilding at position. C-3 (δ 109.0), C-5 (δ 156.8) and C-2' (δ 148.3), confirming the position of hydroxyl groups.

The position of hydroxyl groups were also confirmed on the basis of colour reactions.

1. A deep yellow colour was obtained on treating the compound with ethanolic solution of boric acid in presence of citric acid[17]. This reactions is specific for C-5 -OH group.

2. The compound also gave yellow colour with Zirconium oxychloride (-OH group at C-5)[18].

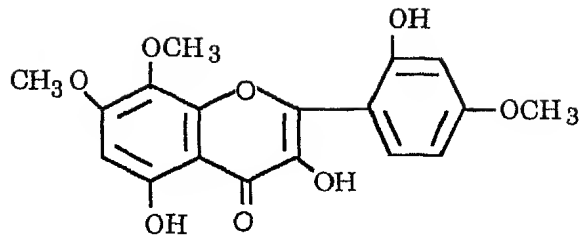
3. The compound gave negative test with vanillin hydrochloric acid reagent which showed the absence of 1,3-dihydroxy group. (no -OH group at C-7).

All positions except the C-7, C-8 and C-4' are filled. ^{1}H NMR spectrum revealed the presence of three methoxyl groups in the aglycone, thus the positions of these methoxyl groups will be at C-7, C-8 and C-4'. The position of methoxyl groups were also confirmed by ^{13}C NMR spectrum by representing signals at δ 158.4 (s, C-7), δ 128.4 (s, C-8) and δ 150.5 (s, C-4'). Mass spectral data confirmed that the presence of two methoxyls and one hydroxyl functions in ring-A and one methoxyl and one hydroxyl group in ring-B[14].

The aglycone does not respond to Gossypetone test[19], which suggested that its C-8 position is blocked. Further the compound does not exhibit any significant change of short wave length band at 265 nm on addition of sodium acetate indicating thereby that one of the methoxyl is located at C-7 position.

Hence aglycone must be 3,5,2'-trihydroxy-7,8,4'-trimethoxy-flavone.

Thus, the structure of aglycone can be written as



^{13}C NMR values are given in Table II.

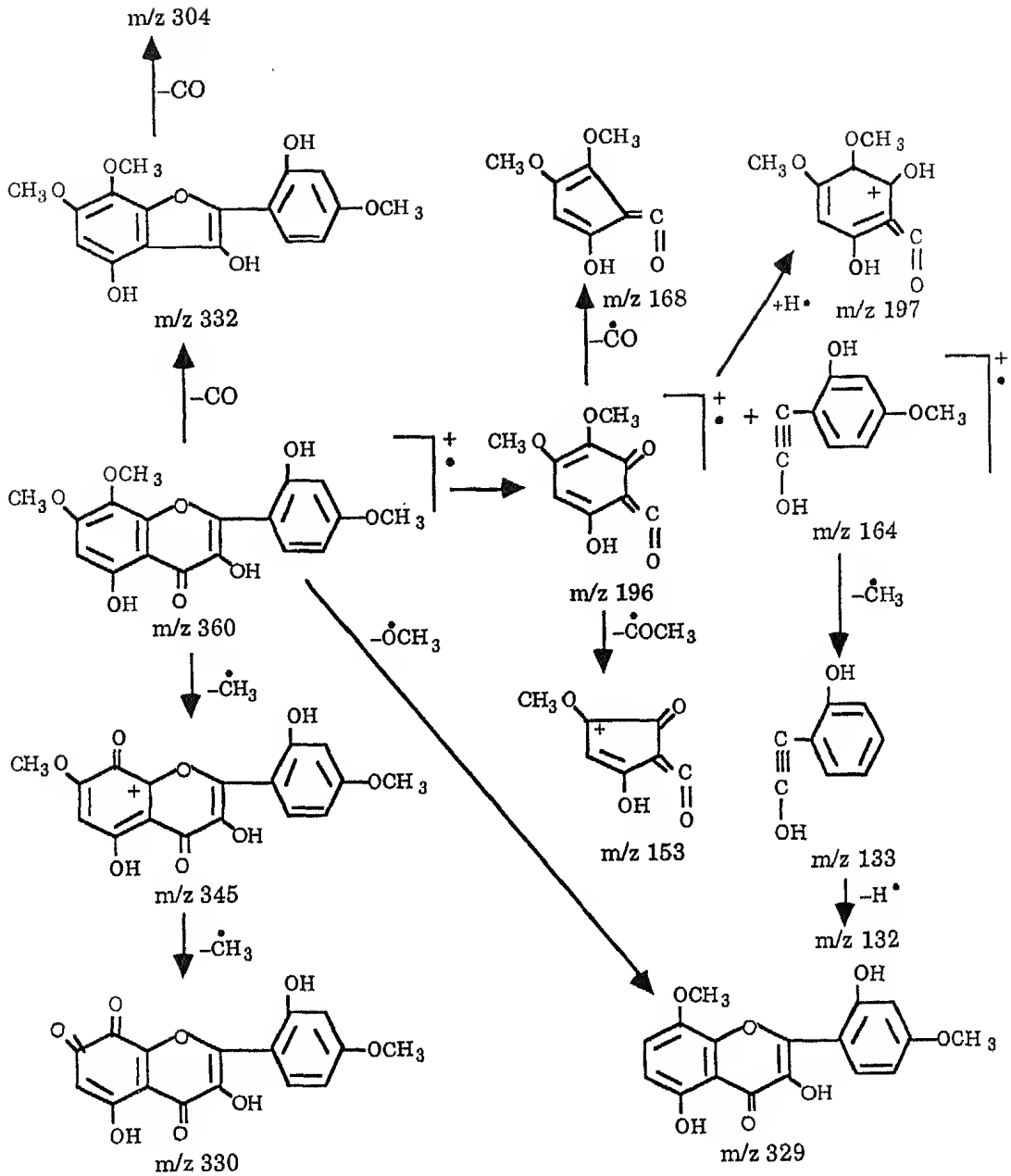
Table - II

Chemical Shifts (ppm)	Splitting	Position Assigned
162.4	s	C-2
109.0	s	C-3
182.0	s	C-4
156.8	s	C-5
96.5	d	C-6
158.4	s	C-7
128.4	s	C-8
148.9	s	C-9
103.8	s	C-10
122.2	s	C-1'
148.3	s	C-2'
107.8	d	C-3'

150.2	s	C-4'
114.6	d	C-5'
119.5	d	C-6'
56.5	q	$-OCH_3$
61.0	q	$-OCH_3$
55.8	q	$-OCH_3$

This structure of the aglycone was further supported by the mass fragmentation pattern of the compound [Chart I]

CHART - I



Glycoside

^{1}H NMR spectral data of the glycoside showed two anomeric proton signals at δ 4.88 (1H, d, J = 5.2 cps) and δ 5.75 (1H, d, J=7.5 cps) ppm, showing that the compound has two sugar moieties. A broad

signal in ^{1}H NMR spectrum at δ 3.10 - 3.85 (12H) indicated the presence of two sugars in the compound which were identified as *D*-galactose and *D*-glucose by direct comparison with their authentic samples. Two doublets at δ 4.88 and 5.75 ppm corresponding to one proton were specific for galactosyl and glucosyl protons[20], respectively. It was further supported by ^{13}C NMR spectral studies.

These two sugars were present either as bioside (disaccharide) and not at different positions as monosaccharide, was confirmed by the mild hydrolysis of the glycoside. It was carried out with 1% aq hydrochloric acid. The course of hydrolysis was progressively followed at regular time intervals by testing the hydrolysate for the two sugars. It was found that glucose unit made its appearance first and it was only after some time that galactose could be detected thereby indicating that glucose was the terminal sugar unit and galactose was directly linked to the aglycone. This confirmed that two sugars were present as bioside. Bioside form of sugars was further confirmed by permethylation of glycoside.

The glycoside was methylated with diazomethane and then subjected to periodate oxidation. One mole of methylated glycoside consumed four moles of periodate which indicated the sugar was present as disaccharide. With the consumption of four moles of periodate in oxidation two moles of formic acid was produced which suggested that sugar was present in pyranose form and not in furanose form.

Quantitative sugar estimation confirmed the presence of two

moles of sugar per mole of glycoside. Easy hydrolysis with β–glucosidase indicated the possibility of C-O-C type of glycosidic linkage between two sugar units. The glycosidic linkage of galactose was not hydrolysed by enzyme. Thus, C-O-C linkage with aglycone was further confirmed by acetylation studies. Aglycone on acetylation gave triacetate and glycoside on acetylation gave nonacetate showing that one hydroxyl group in the aglycone is involved in glycosidic linkage.

Position of sugar linkage

The point of attachment of sugars can be assigned by a comparative study of colour test and spectral shifts of glycoside and aglycone.

The glycoside did not give pink colour with vanillin hydrochloric acid reagent, whereas the aglycone gave positive test with this reagent indicating that one of the hydroxyl at C-5 or at C-7 (ring A) is involved in glycosidation. The glycoside did not give yellow colour with boric acid in presence of citric acid showing that 5-OH group is involve in the glycosidation. This observation was further confirmed by a bathochromic shift of 90 nm (band I) with aluminium chloride and 95 nm with aluminium chloride hydrochloric acid in the UV spectrum of glycoside.

The C-5 site of glycosidation was further confirmed by ^{13}C NMR studies. The ^{13}C NMR spectra of the glycoside (Table III) compared with ^{13}C NMR spectra of aglycone (Table II). The C-5 of the glycoside in ^{13}C NMR spectrum appeared at lower field (161.0 ppm) than C-5 of the

aglycone (156.8 ppm) . Thus C-5 of the glycoside showed deshielding effect, confirmed the attachment of sugars at C-5 of the aglycone through C-O-C linkage.

Inter glycoside linkage in glycoside

The nonreducing nature of glycoside showed that reducing group of both sugars must be involved in linkage formation. Thus, C-1' reducing group of galactose is linked to flavone and C-1" reducing group of glucose is linked to some hydroxyl group of galactose.

The glycoside on permethylation by Hakomori method[21] followed by hydrolysis, yielded 3,4,6-tri-*O*-methyl-*D*-galactose and 2,3,4,6-tetra-*O*-methyl-*D*-glucose which was identified by the method of Jones *et al.* by using 2,3,4,6-tetra-*O*-methyl-*D*-glucose as reference. This showed that inter glycosidic linkage is (1→ 2).

The (1→ 2) interglycosidic linkage was further confirmed by ^{13}C NMR of glycoside. The chemical shifts 79.8 of the C-2" atom of the galactose moiety indicated that C-1"' of *D*-glucose is attached at position C-2" of galactose. It has been revealed that deshielding of C-2" atom on comparison to the reported values for methyl pyranoside confirms (1→ 2) interglycosidic linkage and C-1" of the galactose directly linked with aglycone.

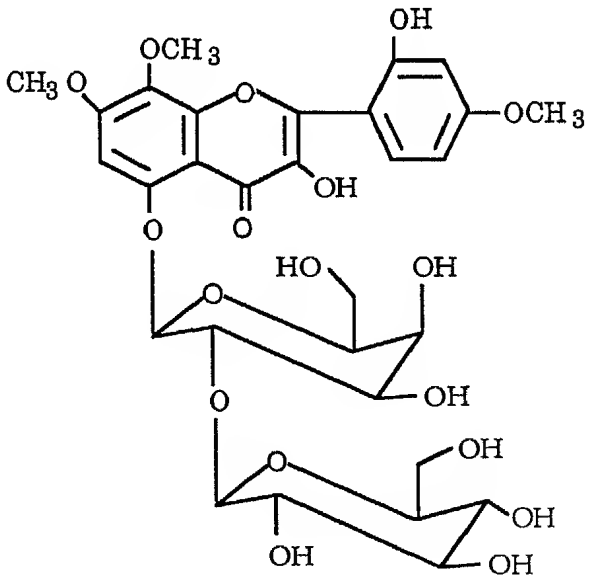
Stereochemical nature of glycoside

Stereochemical nature of sugar linkage was confirmed by enzymatic hydrolysis. When glycoside was hydrolysed with enzyme emulsin it liberated free sugar. Since emulsin is a specific enzyme for

the hydrolysis of β – linkage, so it was concluded that linkage between sugar moiety and hydroxyl group of aglycone was β in nature and sugar-sugar linkage was also β– in nature. The β–nature of glucose and galactose[22] was further supported by its ^{1}H NMR in which its anomeric proton appeared at δ 4.88 (d, 1H, J = 5.2 cps) and δ 5.75 (d, 1H, J = 7.5 cps) respectively.

These observation led to conclusion that C-1" of galactose was involved in the glycosidic linkage with the aglycone and C-1" of glucose involved in interlinkage with C-2" of galactose.

On the basis of above findings, the structure of the compound could be represented as 3,2'-dihydroxy-7,8,4'-trimethoxyflavone-5-*O* [-*β–D-* glucopyranosyl (1→2)] -*β-D*-galactopyranoside.



3, 2'-dihydroxy-7,8,4'-trimethoxy flavone-5-*O*-[-*β-D*-glucopyranosyl(1→2)]-*β-D*-galacto pyranoside

^{13}C NMR spectra of glycoside in Table - III

Table - III

Chemical Shifts (ppm)	Splitting	Position Assigned
162.4	s	C-2
109.2	s	C-3
182.0	s	C-4
161.0	s	C-5
96.5	d	C-6
158.4	s	C-7
128.4	s	C-8
148.9	s	C-9
103.8	s	C-10
122.2	s	C-1'
148.3	s	C-2'
107.8	d	C-3'
150.2	s	C-4'
114.6	d	C-5'
119.5	d	C-6'
98.2	d	gal - C-1"
79.8	d	C-2"
71.9	d	C-3"
67.3	d	C-4"
74.6	d	C-5"
60.8	t	C-6"'
102.2	d	glu - C-1"'
70.0	d	C-2"'
73.6	d	C-3"'
68.2	d	C-4"'
77.0	d	C-5"'
61.9	t	C-6"'

EXPERIMENTAL

The compound was crystallised from methanol

Solublity	:	Chloroform, ethyl acetate and methanol	
m.p.	:	260°C	
Elemental Analysis	:	Found	Calculated for $C_{30}H_{36}H_{18}$
		C-52.39%	C-52.63%
		H-5.31%	H-5.26%
Chromatography	:	Thin layer chromatography was done on silica gel G plates using following solvent system.	
Solvent system	:	benzene - ethyl acetate (4 : 6 v/v)	
R_f	:	0.63	
Spectral studies			
UV λ_{max}^{MeOH} nm	:	255, 360	
$+AlCl_3$	:	275, 450	
$+AlCl_3/HCl$	:	275, 455	

^{1}H NMR : δ 3.85 (s, 3H), 3.95 (s, 3H), 3.98 (s, 3H),

[$CHCl_3$,200MHz] 6.50 (s, 1H), 7.10 (d, 1H, *J*=2 cps), 7.20 (dd, 1H, *J* = 2 & 9 cps), 7.90 (d, 1H, *J* =9 cps), 4.88 (d, 1H, *J* = 5.2 cps), 5.75 (d, 1H, *J* = 7 5 cps), 3.10 - 3.85 (m, 12H, sugar protons) ppm.

^{13}C NMR : δc - 162.4 (s, C-2), 109.2 (s, C-3), 182.0 (s, C-4), 161.0 (s, C-5), 96.5 (d, C-6), 158.4 (s, C-7), 128.4 (s, C-8), 148.9 (s,C-9), 103.8 (s, C-10), 122.2 (s, C-1'), 148.3 (s, C-2'), 107.8 (d, C-3'), 150.2 (s, C-4'), 114.6 (d, C-5'), 119.5 (d, C-6'), 98.2 (d, gal C-1"), 79.8 (d, C-2"), 71.9 (d, C-3"), 67.3 (d, C-4"), 74.6 (d, C-5"), 60.8 (t, C-6"), 102.2 (d, C-1'"), 70.7 (d, C-2'"), 73.6 (d, C-3'"), 68.2 (d, C-4'"), 77.0 (d, C-5'"), 61.9 (t, C-6'") ppm.

Acid hydrolysis of compound

The compound (0.06 g) was refluxed with 7% ethanolic sulphuric acid (55 ml) on a water bath for 4 hr. The reaction mixture was then concentrated, cooled and poured into ice cold water and taken in ether. The aglycone was crystallised from methanol.

Sugar

The hydrolysate was neutralized with barium carbonate filtered and concentrated under reduced pressure. The concentrate gave positive molisch's test, reduced Fehlings solution and gave positive colour test with AHP reagent. It was chromatographed on paper by descending technique using B : A : W (4:1:5, v/v) when sprayed with AHP and heated at 110°C in a oven, revealed two spots (R_f = 0.18 and R_f = 0.20.) suggesting the presence of glucose and galactose. The presence of these sugars was confirmed with co-chromatography with its authentic samples.

Molish's test

The compound (0.05 g) was placed in a test tube containing 0.5 ml of water. It was mixed with 2 drops of a 10% solution of 2-naphthol in ethanol. Conc H_2SO_4 (1 ml) was allowed to flow down the side of the inclined tube so that the acid formed a layer beneath the aqueous solution without mixing with it. A red ring appeared at the common surface of the liquids, the colour quickly changed on standing. On shaking a dark purple solution was formed. The reaction mixture was allowed to stand for two minutes, then diluted with 5 ml of water. A

dull violet precipitate appeared immediately indicating the presence of carbohydrate.

Mild hydrolysis of glycoside

The glycoside (0.03 g) was refluxed with 1% aq hydrochloric acid (10 ml) for 4 hr. Aliquots taken out from time to time from the reaction mixture were neutralised with barium carbonate and tested for free sugars. Glucose was detected after 10 minutes of hydrolysis, while galactose appeared after 30 minutes.

Permethylation of the glycoside

The glycoside (0.03 g) was permethylated by dissolving it in minimum amount of 10% NaOH solution, then dimethyl sulphate (10 ml) was added in several instalment with constant stirring. The temperature of the reaction mixture was maintained at 40-45°C. The solution was concentrated and extracted with acetone. Excess of NaOH was neutralised with dilute sulphuric acid. Methylated product was extracted with $CHCl_3$. Completely methylated glycoside was obtained by evaporating this $CHCl_3$ extract.

Acid hydrolysis of permethylated glycoside

The permethylated glycoside was refluxed with 2N-H_2SO_4 for 4 hr. The hydrolysate was neutralised with barium carbonate, filtered and concentrated under reduced pressure. On paper chromatography (descending) using 2,3,4,6-tetra-*O*-methyl-*D*-glucose as reference sugar confirmed the presence of two partially methylated sugars 3,4,6-tri-*O*-methylgalactose and 2,3,4,6-tetra-*O*-methylglucose by the comparison of R_f values (solvent system, B:A:W, 4:1:5 v/v spray AHP)

Periodate oxidation

Methylated glycoside (0.01g) dissolved in aq ethanol (25 ml, 50%) was added 0.1M sodium meta periodate solution (10ml). A blank experiment was run similarly. Both were kept at room temperature for 48 hr. The molecules of periodate consumed n moles of formic acid liberated were calculated by the titrimetric method of Hirst and Jones. It was observed that for each mole of glycoside.

Moles of periodate consumed = 4.0

Moles of formic acid produced = 2.0

Enzymatic hydroloysis of glycoside

The glycoside in 50% aq ethanol (20 ml) and emulsin solution (10 ml) prepared from almonds were added. The mixture was kept at 40-45°C for 2 hr and then, at room temperature for 4 days. The solution was extracted with ethyl acetate and the remaining hydrolysate was concentrated in a rotary evaporator after neutralization with barium carbonate filteration. The concentrate gave positive. molisch's test and reduces Fehling's solution. On descending paper chromatography gave two spots at different R_f (solvent - system B : A : W, 4 : 1 : 5 v/v spray AHP) identical with that of authentic galactose and glucose, respectively. Co-Pc gave two spots with the same R_f value.

Aglycone

m.p.	:	235°C	
Elemental Analysis	:	Found	Calculated for $C_{18}H_{16}O_8$
		C : 59.3%	C : 60%
		H : 3.0%	H : 3.33%

UV λ_{max}^{MeOH} nm	:	255, 360
$+AlCl_3$	:	255, 420
$+AlCl_3/HCl$	:	255, 420
+ fused NaOAc	:	265, 380
+ NaOAc	:	275, 405
^1H NMR	:	δ 3.85 (s, 3H), 3.95 (s, 3H)
[$CHCl_3$, 200 NHz]		3.98 (s, 3H), 13.25 (broad signal, 1H), 6.50 (s, 1H), 7.10 (d, 1H, *J* = 2 cps), 7.20 (dd, 1H, *J* = 2 & 9 cps), 7.90 (d, 1H, *J* = 9 cps) ppm.
^{13}C NMR	:	δ c 162.4 (s, C-2), 109.0 (s, C-3), 182.0 (s, C-4), 156.8 (s, C-5), 96.5 (d, C-6), 158.4 (s, C-7), 128.4 (s, C-8), 148.9 (s, C-9), 103.8 (s, C-10), 122.2 (s, C-1'), 148.3 (s, C-2'), 107.8 (d, C-3'), 150.5 (s, C-4'), 114.6 (d, C-5'), 119.5 d, C-6'), 56.5 (q, $-OCH_3$), 61.0 (q, $-OCH_3$), 55.8 (q, $-OCH_3$) ppm.

Acetylation

The aglycone (0.05 g) was acetylated using acetic an hydride (5 ml) and pyridine (2 ml) at room temperature for 48 hr. The crude acetate obtained was crystallised from methanol - chloroform. The product obtained was analysed for acetyl groups by elemental analysis.

Colour reactions

1. Ethanolic solution of the compound produced reddish violet colour on addition of magnesium turning and conc hydrochloric acid. (Shinoda test)[3]

2. It also produced red colour when sodium amalgam and hydrochloric acid were added to its ethanolic solution. (Asahina *et al.*)[4]

3. On treatment with conc sulphuric acid, a reddish orange colour with characteristic fluorescence was observed.[5]

4. When exposed to vapours of ammonia, a spot of the compound turned dark yellow, and gave characteristic fluorescence under UV light.[6]

5. Ethanolic solution of the compound on treatment with zirconium oxychloride in presence of citric acid produced a bright yellow colour.[11]

6. A deep yellow colour was obtained on treating the compound with ethanolic solution of boric acid in presence of citric acid. This reaction is specific for a C-5 hydroxyl group.[17]

SECTION – II

Characterisation of compound CO - 2

Apigenin - 7- *O* - β – *D* –allopyranoside

The compound CO-2 m.p. 180°C , (M^+ 432), was analysed for $C_{21}H_{20}O_{10}$. The compound gave positive Molish test[1] showing its glycosidic nature. However, it neither reduced Fehling's solution nor gave a characteristic colour with aniline hydrogen pthalate reagent[2], indicating that the reducing group of sugar was not free and was involved in glycosidic linkage.

On acid hydrolysis, compound gave an aglycone and a sugar. The sugar was identified as D-allose by co-paper chromatography with an authentic sample.

Aglycone

The aglycone, (M^+270), m.p. 139°C was found to have molecular formula $C_{15}H_{10}O_5$. It responded to the following colour reactions -

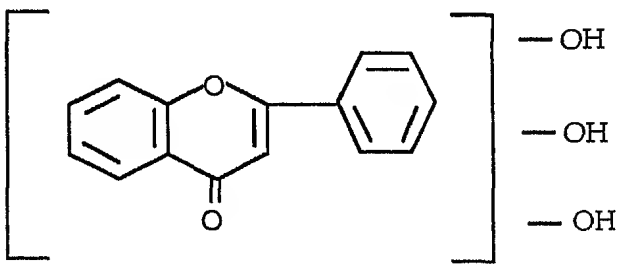
1. Olive green colour was obtained when the compound was treated with 1% ethanolic ferric chloride solution[8].
2. Its alcoholic solution gave pink colour with magnesium turnings and conc. hydrochloric acid [3].
3. Pink colour was obtained on treating its alcoholic solution with sodium amalgam and dilute hydrochloric acid[4].
4. It gave a dark yellow spot on a filter paper when exposed to ammonia vapours[6].
5. It gave a yellowish orange solution with characteristic fluorescence, when treated with conc. sulphuric acid[5]. These colour reactions confirmed the presence of a flavonoid skeleton, possibly a flavonol or flavonone nucleus.

The compound did not give a positive colour reaction with 2, 4-dinitro phenylhydrazine[23]. It could not be reduced with sodium borohydride[24], thus ruling out the possibility of its being a flavonone.

The compound did not give any colour with zirconium oxychloride[25] in presence of citric acid[11,26,27], neither did it give a positive colour reaction with boric acetate and sodium acetate, thus ruling out the possibility of it being a flavonol.[28,29]

On acetylation with acetic anhydride and fused sodium acetate it gave triacetate showed the presence of three hydroxyl groups in the compound . The ^{1}H NMR spectrum of flavone showed the presence of seven protons in aromatic region, indicating that compound is trisubstituted flavone which was also supported by ^{13}C NMR.

The compound can thus be represented as :



This possible structure accounted for all the carbons, hydrogen and oxygen atoms. The relative position of the hydroxyl group was further ascertained on the basis of colour reactions and spectral data.

Position of - OH groups

The compound showed following colour reaction :

1. It gave bright yellow colour with boric acid in presence of citric acid.[30]

2. Spot test with 1% ethanolic $AlCl_3$ showed greenish yellow fluorescence under UV light [31,32].

3. It gave a pink colour with 3% p-toluene sulphonic acid.[33,34]

4. Pink colour was also obtained with boric acid and acetic anhydride.[35]

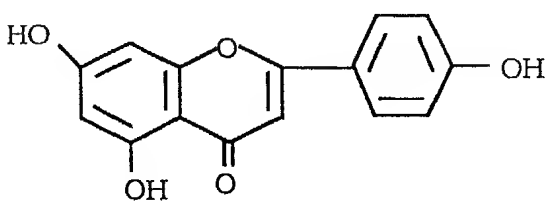
5. The compound also gave yellow colour with zirconium oxychloride.[18]

All these colour reactions specific for the presence of hydroxyl group at C-5 in a flavone, further confirmed the C-5 position of -OH group.

The compound was soluble in aqueous sodium carbonate, provided evidence for the presence of phenolic groups at C-7 and C-4' position.[8]

The presence of 5,7 hydroxyl groups were confirmed by positive test with vanillin hydrochloric acid reagent (reagent for 1,3-dihydroxy group)[36].

The ^{13}C NMR spectrum of the compound showed singlet at δ 161.1, 159.2 and 161.3 ppm indicated the presence of hydroxyl group at C-5, C-7 and C-4', respectively . Thus aglycone is 5,7,4'-trihydroxy flavone.



The ^1H NMR spectrum of the aglycone exhibited two signals at δ 6.17 (d, 1H, J = 2.2 Hz) and 6.46 (d, 1H, J = 2.2 Hz) were due to protons at C-6 and C-8, respectively. Coupling constant value of these protons showed the signals for two meta coupled protons. Signals at δ 7.90 (d, 1H, J = 8.9 Hz), 6.92 (d, 1H, J = 8.9 Hz), 6.92 (d, 1H, J = 8.9 Hz) and 7.90 (d, 1H, J = 8.9 Hz)ppm were assigned four meta coupled protons at C-2', C-3', C-5' and C-6', respectively. ^1H NMR showed singlet at δ 6.72 (1H) ppm for proton at C-3. One proton singlet at δ 12.92 due to chelated phenolic hydroxyl group.

These results further confirmed the presence of hydroxyl groups at position C-5, C-7 and C-4' of compound. This structure was also supported by its ^{13}C NMR.

Table - II

^{13}C NMR (At 75.46 MHz in DMSO-d_6)

Chemical Shifts (ppm)	Splitting	Position Assigned
163.6	s	C-2
102.7	d	C-3
181.5	s	C-4
161.1	s	C-5
98.7	d	C-6
162.2	s	C-7
93.8	d	C-8
157.2	s	C-9
103.5	s	C-10
121.1	s	C-1'

128.3	d	C-2'
115.8	d	C-3'
161.3	s	C-4'
115.8	d	C-5'
128.3	d	C-6'

Table - I

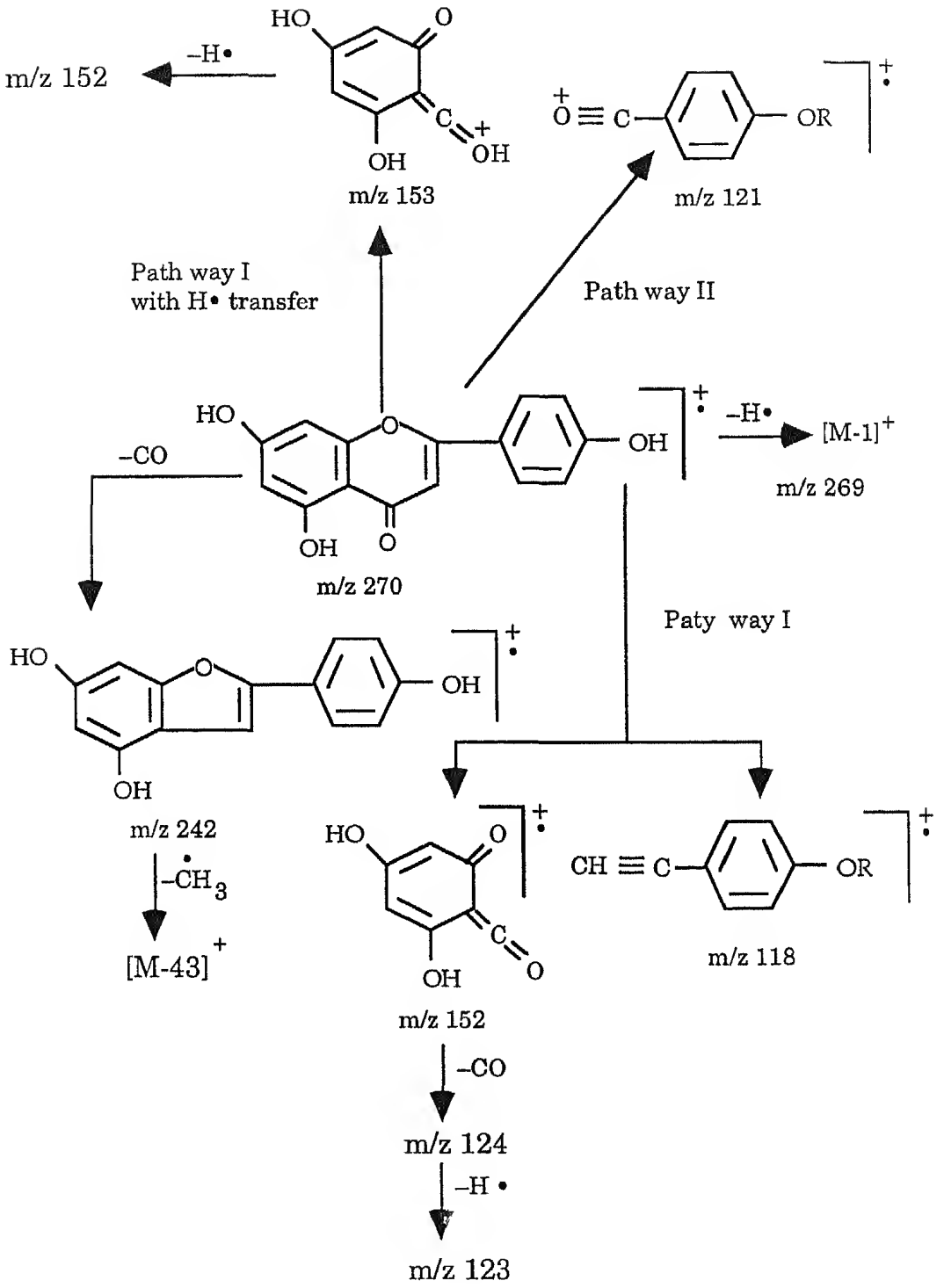
^{1}H NMR (300 MHz in DMSO-d_6)

Chemical shift (ppm)	*J* value (Hz)	Assignment
6.72 (1H, s)		C-3 proton
6.17 (1H, d)	2.2	C-6 proton
6.46 (1H, d)	2.2	C-8 proton
7.90 (1H, d)	8.9	C-2' proton
6.92 (1H, d)	8.9	C-3' proton
6.92 (1H, d)	8.9	C-5' proton
7.90 (1H, d)	8.9	C-6' proton
12.92 (1H, s)		C-5 proton

Mass spectral studies

The mass fragmentation studies confirmed the structure of the compound.

Chart - I


m/z 152
–H•
HO
O
OH
OH
m/z 153
+O≡C
OR
m/z 121
Path way I
with H• transfer
Path way II
HO
O
OH
–H•
[M-1]+
m/z 269
–CO
OH
O
m/z 270
Paty way I
HO
O
OH
OH
m/z 242
–CH3
[M-43]+
HO
O
OH
O
m/z 152
CH ≡ C
OR
m/z 118
–CO
m/z 124
–H •
m/z 123

Glycoside

The glycoside $C_{21}H_{20}O_{10}$ (M^+432), m.p. 180°C, on acid hydrolysis gave aglycone apigenin and sugar where it was identified as D-allose by co-chromatography with an authentic sample. The presence of allose was supported by signals in ^1H NMR at δ 5.75 (d, 1H, J = 7 Hz, allosyl H-1') and ^{13}C NMR spectrum at δ 77.1, 74.6, 70.9, 67.3 and 60.8 ppm. Quantitative sugar estimation confirmed the presence of one mole of sugar per mole of glycoside.

Position of attachment of sugar to aglycone

The point of attachment of sugar moiety to the aglycone was confirmed by a comparative study of the colour reaction of the glycoside and the aglycone.

1. The glycoside did not give pink colour with vanillin hydrochloric reagent[6].
2. The aglycone gave positive test with this reagent. This reaction indicated that one of the hydroxyl group at C-5 or at C-7 is involved in glycosidation.
3. The glycoside gave yellow colour with boric acid[37] in presence of citric acid and yellow colour with zirconium oxychloride indicating that -OH group at C-5 is free.

This reaction indicated that hydroxyl group at C-7 is involved in the glycosidation. The site of glycosidation was found to be at C-7 on the basis of UV shift with NaOAc and ^{13}C NMR[38] spectral data. When the ^{13}C NMR spectrum of the glycoside was compared with that of the

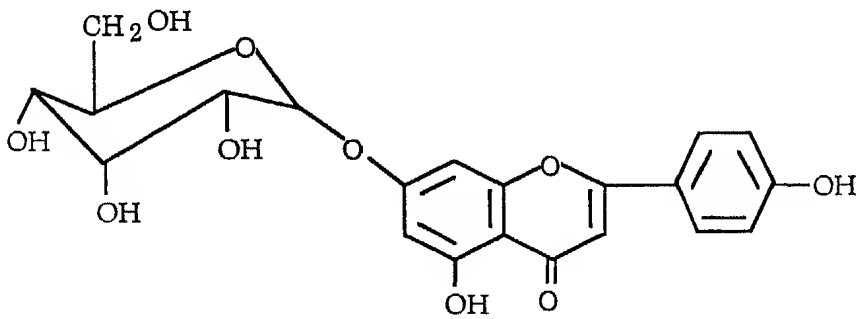
aglycone, the glycoside showed the downfield signal for C-7 at δ 164.2 ppm which appeared at δ 162.3 ppm in the aglycone. This confirmed the C-7 position for the attachment of allose to the aglycone.

Stereochemical nature of sugar linkage

Stereochemical nature of the sugar linkage was confirmed by enzymatic hydrolysis. Emulsin, being specific enzyme for *β*– linkage hydrolysed the glycoside. The free sugar could be detected in the hydrolysate, so it was concluded that the linkage between sugar moiety and aglycone is *β*- in nature.

The signal in the ^{1}H NMR of the glycoside at δ 5.75 (d, 1H, J = 7.5 Hz, allosyl H-1) suggested *β* - linkage of D-allose with the aglycone. Easy hydrolysis eliminated the possibility of C-C glycosidic linkage so the linkage must be of C-O-C type. The ^{1}H NMR spectrum of compound showed signal at δ 3.58 (m, sugar protons) ppm and ^{13}C NMR spectrum suggested the sugar moiety is D-allose.

On compiling all the evidences, the compound CO-2 was identified as apigenin-7-*O*-*β*-*D*-allopyranoside and was represented as :



apigenin-7-*O*-*β*-*D*-allopyranoside

The above glycoside is new and was not reported earlier from any plant source.

EXPERIMENTAL

The compound was crystallised from methanol.

Solubility	:	Chloroform, ethyl acetate and methanol	
m.p.	:	180°C	
Elemental analysis	:	Found	Calculated for $C_{21}H_{20}O10$
		C : 47.36%	C : 47.25%
		H : 6.57%	H : 6.60 %
Chromatography	:	TLC was done on silica gel G plates using following solvent system and spray reagent.	
Solvent system	:	benzene - ethyl acetate (7 : 3 v/v)	
R_f	:	0.50	
Spectral Studies			
UV λ_{max}^{MeOH}	:	268, 333 nm.	
^1H NMR	:	δ 6.72 (s, 1H, C-3), 6.17 (d, 1H, *J* = 2.2 Hz, C-6),	
[at 300 MHz in		6.46 (d, 1H, *J*= 2.2 Hz, C-8), 7.90 (d, 1H, *J* = 8.9	
DMSO-d_6]		Hz, C-2'), 6.92 (d, 1H, *J* = 8.9 Hz, C-3'), 6.92 (d, 1H, *J* = 8.9 Hz, C-5'), 7.90 (d, 1H, *J* = 8.9 Hz, C-6'), 12.92 (s, 1H, C-5 hydroxy group), 5.75 (d, 1H, *J* = 7.5 Hz, allosyl H-1'), 3.58 (m, 6H, sugar protons) ppm.	
^{13}C NMR	:	δ 163.6 (s, C-2), 102.7 (d, C-3), 181.5 (s, C-4),	
[at 75.46 MHz in		161.1 (s,C-5), 98.7 (d, C-6), 164.2 (s, C-7), 93.8	
DMSO-d_6]		(d, C-8), 157.2 (s, C-9), 103.5 (s, C-10), 121.1 (d, C-	

1'), 128.3 (d, C-2'), 115.8 (d, C-3'), 161.3 (s, C-4'), 115.8 (d, C-5'), 128.3 (d, C-6'), 98.2 (d, C-1''), 76.1 (d, C-2''), 74.6 (d, C-3''), 67.39 (d, C-4''), 74.6 (d, C-5''), 60.8 (t, C-6'') ppm.

Acid hydrolysis of compound

The glycoside (0.05 g) was refluxed with 50 ml of 7% ethanolic sulphuric acid on a water bath for 4 hr. The reaction mixture was then concentrated, cooled and poured into ice-cold water whereby a solid mass got separated which was extracted with ether and crystallised from methanol.

Sugar

The remaining aqueous solution was neutralized with barium carbonate and filtered. The filtrate was concentrated on a rotary evaporator. The concentrate gave positive Molisch's test, reduced Fehling's solution and gave positive colour test with AHP reagent. It was chromatographed on paper by descending technique using B : A : W (4 : 1 : 5, v/v). When sprayed with AHP and heated at 110°C in an oven, it revealed one spot (R_f = 0.18) identical with the authentic allose sample.

Molisch's test

Positive Molisch's test indicated the presence of carbohydrate. Test was performed as described earlier on page no. 91

Aglycone

Elemental Analysis

Found	Calculated for $C_{15}H_{10}O_5$
C : 66.58%	C : 66.67%
H : 3.52%	H : 3.70%

Acetylation

The compound (0.05g) was acetylated with acetic anhydride (4ml) and pyridine (2.0ml) at room temperature for 48 hr, then poured over crushed ice and left overnight. The solid obtained was filtered, washed with cold water and crystallized from ethyl acetate - petroleum ether mixture. The acetyl percentage in the acetylated product was determined by elemental analysis.

Found : $-COCH_3$ = 32.49%

Calculated for $C_{15}H_7O_5(COCH_3)_3$ = 32.57%

Spectral Studies:

^{13}C NMR [at 75.46 MHz in DMSO-d_6]	:	δ 163.6 (s, C-2), 102.7 (d, C-3), 181.5 (s, C-4), 161.1 (s, C-5), 98.7 (d, C-6), 162.3 (s, C-7), 93.8 (d, C-8), 157.2 (s, C-9), 103.5 (s, C-10), 121.1 (d, C-1'), 128.3 (d, C-2'), 115.8 (d, C-3'), 161.3 (s, C-4'), 115.8 (d, C-5'), 128.3 (d, C-6') ppm.
Mass spectra m/z	:	270 (M^+), 269, 242, 227, 153, 152, 124, 123, 121, 118.

Enzymatic hydrolysis of glycoside

Emulsin hydrolysis

The glycoside in 50% aq. ethanol (20 ml) and emulsin solution (10 ml) prepared from almonds were added. The mixture was kept at 40-45°C for 2 hr and then at room temperature for 4 days. The mixture was then extracted with ethyl acetate and the remaining aqueous solution was concentrated in a rotary evaporator to a syrup. The syrup on PC (descending) gave single spot, R_f 0.18 (solvent, B : A : W, 4:1:5, v/v, upper layer, spray AHP). Co-chromatography with an authentic sample gave only one spot.

SECTION - III

Characterisation of compound CO-3

2α, 3β, 19α, 23–tetrahydroxy- urs -12-en-28-oic acid

The compound CO-3, m.p. 283-285°C, established its molecular formula as $C_{30}H_{48}O_6$, on the basis of elemental analysis and molecular weight determination (M^+504). The compound gave following colour reactions characteristic of triterpenoids.

1. It gave yellow colour changing to red in salkowski reaction[39].
2. A deep red colour in Liebermann - Burchard reaction[40].
3. A red colour with a greenish fluoresence in Tschugajew reaction[41].
4. The solution of the compound developed red colour on treating with Noller's reagent[42].
5. A reddish colour with Rosenheim reagents[43].
6. A deep purple colour in Kohlenberg's reaction[44].
7. The compound produced a reddish violet colour in Brieskorne test[45].

The compound gave violet colour with 2,6- di-tert-butyl p-cresol in ethanol[46], indicating it to be a pentacyclic triterpene. It's ^{13}C NMR spectrum of 30 signals also indicated that compound is triterpenoid.

The absorption peak in IR spectrum of compound at 3300 cm^{-1} showed the presence of hydroxyl group. The peak at 3500 cm^{-1} is due presence of tertiary hydroxy group. The peaks at 2933, 1381 and 1362 cm^{-1} indicates the presence of C-CH_3 group.

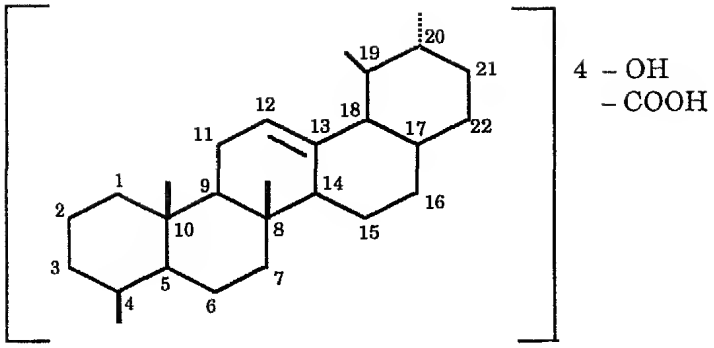
Compound on acetylation forms triacetyl derivative. IR spectrum of acetyl derivative shows peaks at 3500 cm^{-1}. This confirmed

that tertiary hydroxyl group is not acetylated. This compound has four hydroxyl group.

Acetylated compound on methylation with diazomethane provided its monomethyl ester indicating the presence of one carboxylic group.

The presence of carboxyl group was further confirmed by peak at 1690 cm^{-1} in the IR spectrum of compound and a band at 1720 cm^{-1} in the IR spectrum of mono methylester. The molecular weight (M^+518) of mono methylester confirms the presence of only one COOH group in the compound. ^1H NMR of compound showed a characteristic broad singlet at δ 2.52 (1H, 18β -H) and exhibited signals between δ 0.70 – 1.34 (s, 5 x 3 H) for five tertiary methyl group and at δ0.95 (d, 3H, J = 6.5 Hz) for one secondary methyl group. A triplet appeared at δ5.32 (1H) for C-12 olefinic proton, showed the presence of double bond at 12:13 position of amyrin nucleus. It was further confirmed by colour with tetranitromethane (Ruzicka reaction)[47] typical of 12:13 double bond present in triterpenes of α and β amyrin series.

Thus the above facts conclusively indicated that the compound to be a pentacyclic unsaturated triterpene of amyrin series with four hydroxyl and one carboxyl group. Thus, the structure can be represented as :



Position of -COOH group

The position of -COOH group at C-17 in the compound was confirmed by a signal at δ 3.60 (s, 3H, - $COOCH_3$) ppm in ^1H NMR of methyl ester.

Position of OH groups

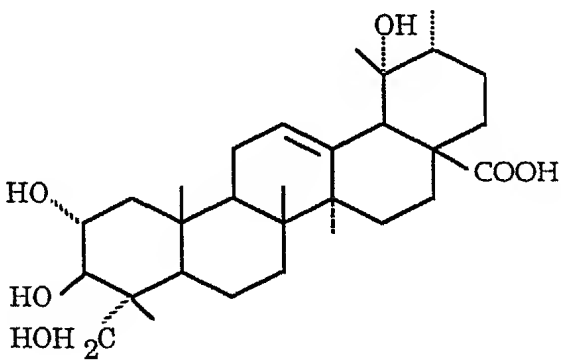
The ^{13}C NMR of compound CO-3 is same as that of 2α, 3β, 23–trihydroxyolean-12-en-28-oic acid (arjunolic acid)[48] with regard to signals assignable to C-2, C-3, C-12, C-13, C-23 and C-28. These data suggested that compound is a derivative of arjunolic acid having one tertiary or hindered hydroxyl group. A singlet at δ 2.57 in ^1H NMR spectrum of compound suggested presence of 19α– hydroxy group. The ^{13}C NMR spectrum also resembled with 19α – hydroxyursolic acid[49] with regards to signals assignable for C-12, C-13, C-18, C-19, and C-28.

The compound gave positive Zimmermann test suggesting the C-3 position of hydroxyl group, which was biogenetically favoured. The position of remaining two hydroxyl group were also confirmed by spectral studies of acetyl derivative of compound. The ^{13}C NMR of compound on comparing with arjunolic acid showed the presence of

two secondary hydroxyl groups at adjacent positions, C-2 and C-3. In ^{13}C NMR spectra it was found that C-23 appeared as triplet at δ 66.4 and at C-24 as quartet at δ 14.2 ppm. The high δ value of C-23 was due to presence of -OH group. This conclusively confirmed that C-23 was present in form of hydroxymethyl at C-4 position. C-23 having $-CH_2OH$, showed anisotropic effect corresponding to two protons at δ 3.57 (d, 1H, J = 12 Hz) and at δ 3.84 (d, 1H, J = 12 Hz) ppm in different environment.

The ^1H NMR spectrum had signals at δ 5.05 [(d,1H, J = 10 Hz, at C-3 (α-OH)] and 5.20 [(m, 1H, at C-2 (β-OH)] ppm due to hydroxymethine protons. The splitting pattern of these protons suggested that one hydroxyl group was axial and one was equatorial.

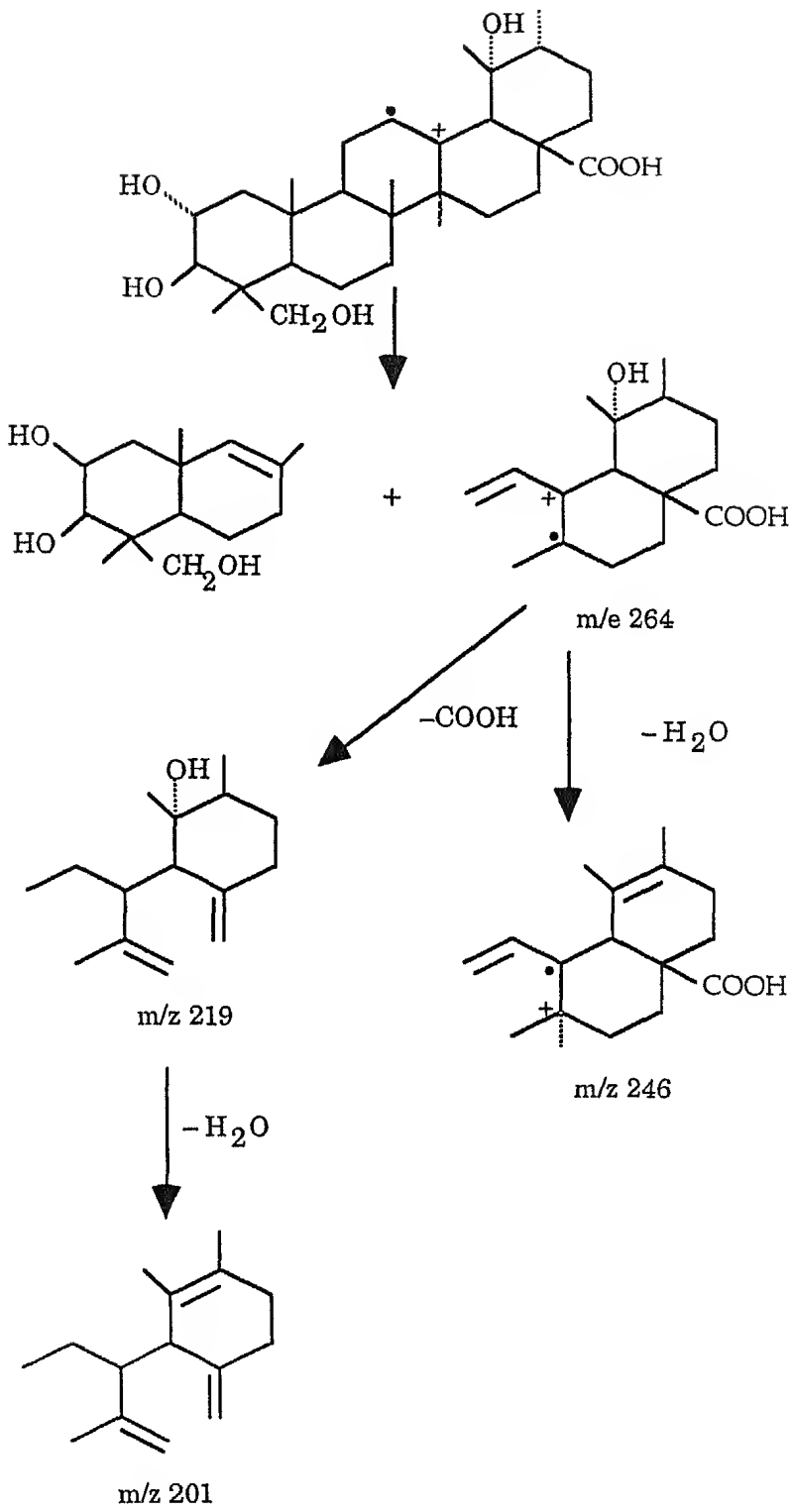
Accordingly, the compound must be 2α, 3β, 19α, 23-tetrahydroxy urs-12-en-28-oic acid (19α- hydroxy asiatic acid)[50] which was confirmed by co-chromatography with authentic sample[51].



2α , 3β, 19α, 23-tetrahydroxy urs-12-en-28-oic acid

This structure was further supported by its mass spectrum. The mass spectrum showed characteristic fragment peaks at m/z 504 [H^+], 264, 246, 219 and 201 due to retro-Diels Alder cleavage of urs-12-en-28-oic acid derivatives bearing one hydroxyl group in the D- or E- ring.

Chart – I


OH
COOH
HO
HO
CH_2OH
HO
HO
CH_2OH
+
OH
COOH
m/e 264
-COOH
$-H_2O$
OH
m/z 219
COOH
m/z 246
$-H_2O$
m/z 201

It was crystallised from methanol.

m.p.	:	283-285°C
R_f	:	0.60 (1:1 v/v, $CHCl_3$: MeOH)
Colour reactions	:	The compound gave following colour reactions.

1. Salkowski reaction [39]

When concentrated sulphuric acid was added to a solution of the compound in chloroform, a yellow colour changing to red was observed.

2. Liebermann - Burchard reaction [40]

The compound was dissolved in acetic anhydride and a few drops of concentrated sulphuric acid were added to it. A red violet colour was developed.

3. Tschugajew reaction [41]

When a solution of the compound in chloroform was mixed with acetyl chloride and zinc chloride and boiled, a violet red colour was developed.

4. Noller's reaction [42]

When a little of the compound was treated with Noller's reagent (prepared by adding 0.01% stannic chloride in pure thionyl chloride) a light pink colour changing to violet was observed.

5. **Brieskorne reaction** [45]

When a little of the compound was treated with 30% solution of chlorosulphonic acid in glacial acetic acid, it turned reddish violet.

6. **Ruzicka test** [47]

It gave yellow colour with tetranitromethane.

Elemental Analysis :

Found	Calculated for $C_{30}H_{48}O_6$
C : 71%	C : 71.4%
H : 9.6%	H : 9.5%

IR ν_{max}^{KBr} : 3500, 3300, 2933, 1720 (ester), 1690, 1381, 1362 cm^{-1}.

Mass spectra m/z : 504[M^+], 264, 246, 219, 201.

^1H NMR [$(CD_3)_2CO$,90 MHz] : δ 0.70-1.34 (s, 15H, 5-CH_3 group), 0.95 (d, 3H, J = 6.5 Hz at C-30), 5.32 (t, 1H, C-12 olefinic proton), 2.57 (s, 1H, at C-19α-OH group), 2.52 (s, 1H, 18 β H), 3.57 and 3.84 (each d, 1H, J = 12 Hz, at C-23), 5.05 (d, 1H, J = 10 Hz, at C-3), 5.20 (m, 1H, at C-2) ppm.

^{13}C NMR : δ_c 47.7 (t, C-1), 68.8 (d, C-2), 78.4 (d, C-3), 43.5 (s, C-4), 48.5 (d, C-5), 18.8 (t, C-6), 33.1 (t, C-7), 40.6 (s, C-8), 48.0 (d, C-9), 38.4 (s, C-10), 24.2 (t, C-11), 127.5 (d, C-12), 139.1 (s, C-

13), 42.1 (s, C-14), 29.1 (t, C-15), 26.0 (t, C-16), 49.6 (s, C-17), 54.3 (d, C-18), 72.3 (s, C-19), 42.6 (d, C-20), 26.7 (t, C-21), 37.6 (t, C-22), 66.4 (t, C-23), 14.2 (q, C-24), 17.4 (q, C-25), 17.4 (q, C-26), 24.5 (q, C-27), 180.2 (s, C-28), 27.2 (q, C-29), 17.0 (q, C-30) ppm.

Acetylation of compound

The compound was acetylated with acetic anhydride and pyridine at 80°C for 1.5 hr. Crystallisation from ethanol gave colourless needles of monohydroxy acetate.

m.p.	:	210 - 212°C
Elemental analysis	:	
Found		Calculated for $C_{36}H_{54}O_9$
C: 68.7%	:	C : 68.6%
H : 8.6%		H : 8.6%
Mass spectra m/z	:	630 [M^+]
^1H NMR	:	δ 0.70 - 1.34 (s, 15, 5 x CH_3 group), 0.95 (d,
[$CDCl_3$ 90 MHz]		3H, J = 6.5 Hz, at C-30), 1.99, 2.04, 2.10, (each s, 3H, OAc x 3), 2.52 (s, 1H, 18 β H,) 3.57 and 3.84 (each d, 1H, J = 12 Hz, at C-23), 5.05 (d, 1H, J = 10 Hz, at C-3), 5.20 (m, 1H, at C-2), 5.32 (t, 1H, C-12 olefinic

proton) ppm.

Methylation of acetylated product

The compound (1.0g) was treated with ethereal solution of diazomethane till a permanent yellow colour was obtained. The contents were cooled in ice during the addition of ethereal solution of diazomethane. The excess of diazomethane was destroyed by the addition of acetic acid. The ethereal solution was washed first with aqueous $NaHCO_3$ and then with water. It was dried over anhydrous sodium sulphate and concentrated, whereupon a white residue was obtained. It was crystallised from methanol gave mono methylester.

^1H NMR ($CDCl_3$, 90 MHz) : δ 3.60 (s, 3H, -$COOH_3$ at C-17)ppm.

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CHAPTER - 3

CHEMICAL EXAMINATION OF THE FRUIT PERICARP OF *Feronia limonia*

The air-dried and finely crushed fruit pericarp (5 kg) of *Feronia limonia* was extracted with ethanol. The ethanolic extract on keeping over night, deposited a dirty white residue (compound FL-4), which was separated by filteration. The deposit was washed several times with cold ethanol and dried over calcium chloride in a vacuum desiccator. It was kept for further purification.

The filterate after the separation of the flocculent deposit was further concentrated and poured into large excess of ice cold water with constant stirring. A reddish brown aqueous solution and a dark brown water insoluble residue was separated.

Water soluble fraction

The water soluble fraction, concentrated in a rotary evaporator, and fractionated by liquid-liquid extractor with different solvents in increasing order of polarity, viz, hexane, benzene, ether and ethyl acetate, respectively.

From the hexane fraction practically nothing could be detected. The benzene extract was concentrated and allowed to stand when a white crystalline substance separated out after 3-4 hr, which after crystallisation from pure methanol, yielded a white shining needle like compound, FL-1 m.p. 189°C. Its homogeneity and purity was checked by thin layer chromatography.

From ethyl acetate extract, on keeping for about two days at low temperature, after concentration, a white compound FL-2, m.p. 262°C, was isolated after repeated crystallisation.

Water insoluble fraction

The water insoluble fraction was successively extracted with hexane, benzene, ether and ethyl acetate in a soxhlet extractor.

Single compound has been isolated by means of preparative TLC from benzene soluble fraction, viz., FL-3 (m.p. 66°C)

Thus, four compound have been isolated and studied in detail. They are identified as :

Compound FL-1 : 2'-hydroxyisopropyldihydrofuranocoumarin

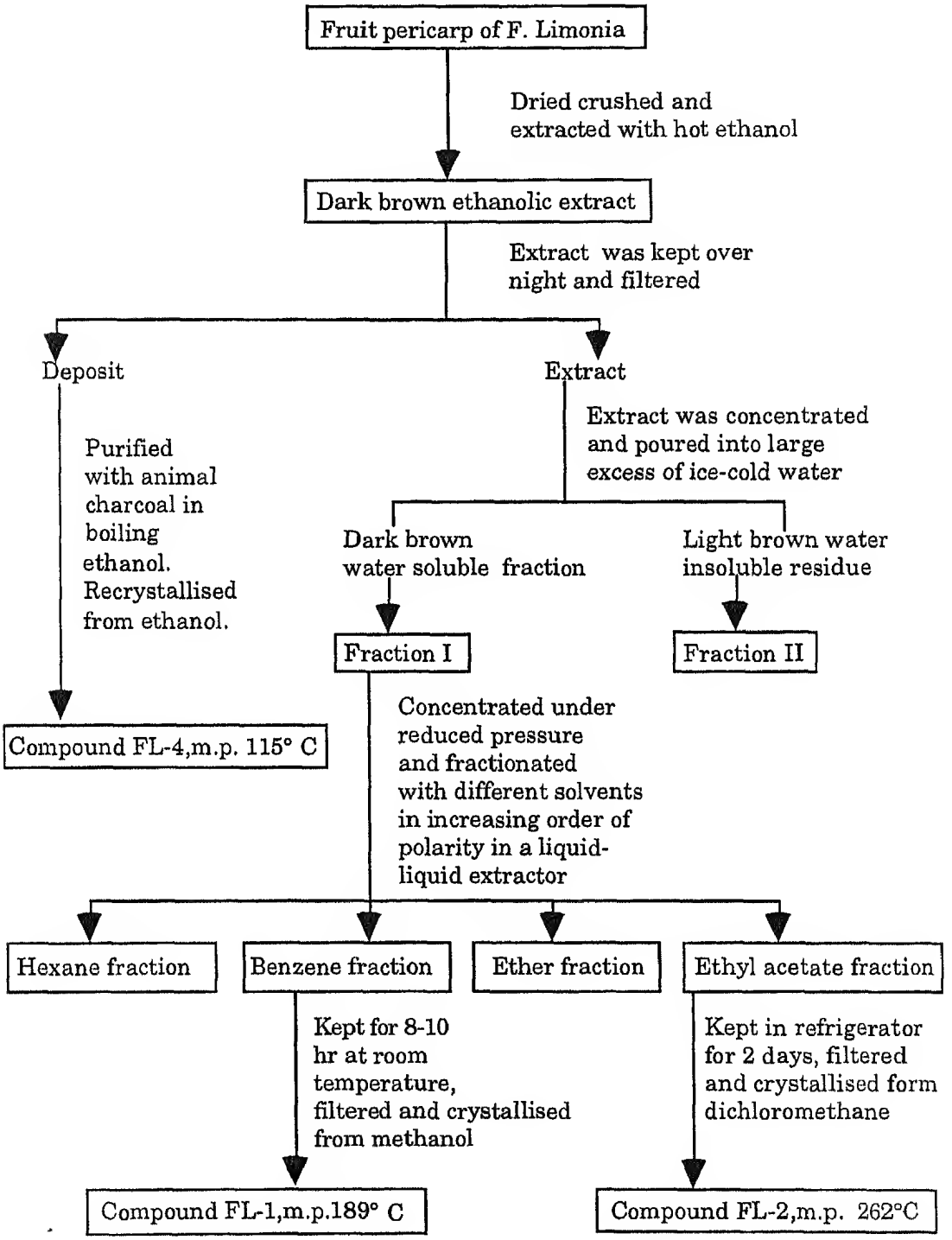
Compound FL-2 : Fernolin

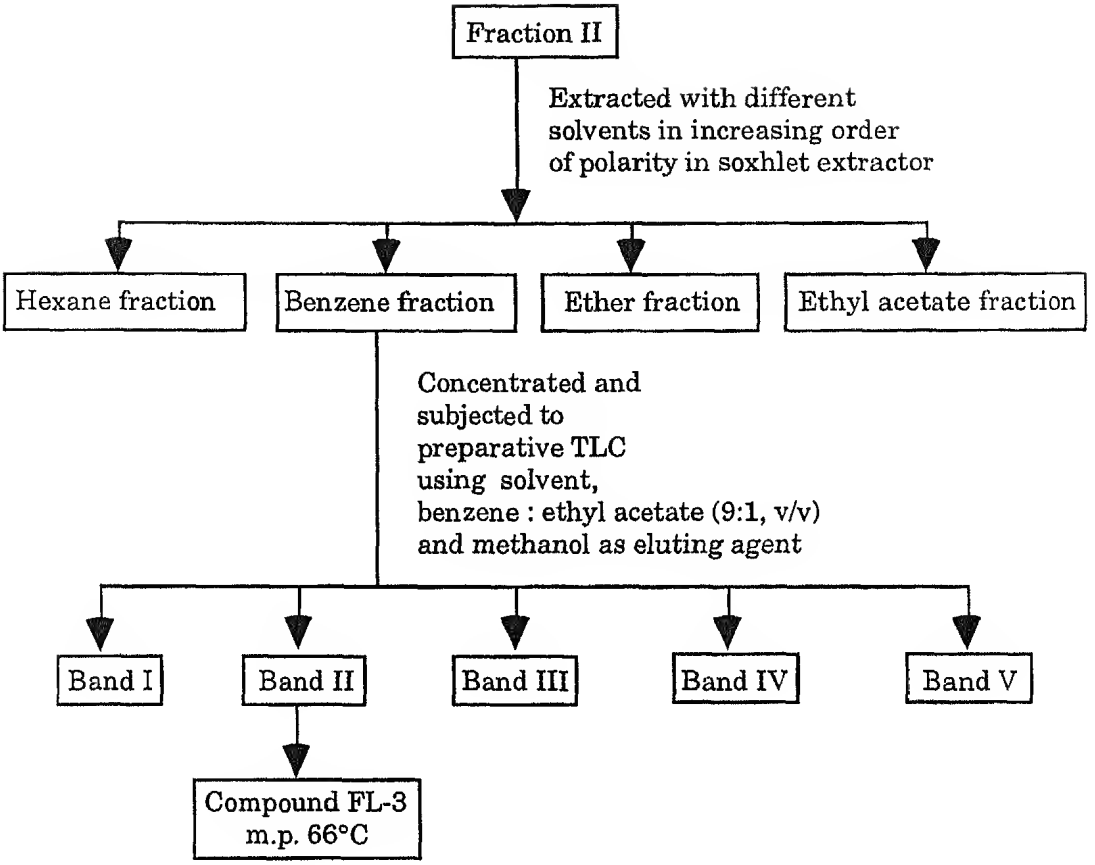
Compound FL-3 : 7-geranyloxycoumarin

Compound FL-4 : Feronolide

Detailed structural studies of these compounds are given is section I, II, III, and IV of this chapter, respectively

Scheme of isolation and purification of compound from the fruit pericarp of *Feronia limonia*





Experimental

The fruit pericarp of *Feronia limonia* was collected from the Pratapgarh, U.P. Its identity was confirmed with the help of Botanical Survey of India, Allahabad.

Extraction with ethanol

The air - dried and finely crushed fruit pericarp of *F. Limonia* (4kg) was exhaustively extracted with ethanol (5×2.5 litres) under reflux in a 5 litre flask. The alcoholic extract on keeping over night, deposited a flocculent dirty white precipitate which was filtered off under suction. Thus, compound FL-4 was obtained.

Purification of FL-4

The crude compound was refluxed with large excess of absolute ethanol and filtered through a hot water funnel. A cream coloured compound was deposited on cooling the filtrate and thus a very large excess of adhering colouring matter and a little chlorophyll was removed. The process was repeated several times. Then the product was refluxed with absolute ethanol and a little amount of animal charcoal for about eight hours and was filtered off. The filterate now deposited a residue which was creamish white and free from chlorophyll and other colouring impurities. It was filtered and washed with absolute ethanol and ether. The above process was repeated several times, when a creamish white compound was obtained i.e. compound FL-4 m.p. 115°C. It was dried over calcium chloride in a vaccum desiccator.

The remaining portion of alcoholic extract was concentrated (250ml) under reduced pressure in rotary evaporator and poured into a large excess of ice-cold water (1.5 litre) with constant stirring, whereby the dark brown water soluble (fraction I) and light brown water insoluble (fraction II) portions were separated out. Each fraction was worked out separately.

Fraction I

The water soluble fraction was concentrated under reduced pressure (200 ml) and was fractionated by liquid-liquid extractor with hexane, benzene, ether and ethyl acetate, respectively.

From hexane and ether fractions practically no compound could be obtained.

From, the benzene soluble fraction, on keeping for 8-10hr at room temperature, a white substance was settled down which was separated by filteration. The substance was crystallised in white shining needles from methanol, FL-1, m.p. 189°C (2.0g).

From the ethyl acetate fraction another compound was separated out. On keeping the fraction in refrigerator for 2 days, a white compound was settled down at the bottom of the flask, which was separated by filteration and crystallised from dichloromethane, FL-2, m.p. 262°C (600mg).

Fraction II

The water insoluble fraction was successively extracted with hexane, benzene, ether and ethyl acetate in a soxhlet extractor Each fraction was worked out separately.

From hexane, ether, and ethyl acetate fractions practically nothing could be obtained.

Benzene fraction gave single compound. The benzene fraction was concentrated under reduced pressure. The concentrate revealed the presence of five constituents on TLC. It was subjected to preparative TLC using solvent, benzene : ethyl acetate (9:1, v/v). Band were marked with the help of UV lamp. All the five bands were collected separately and extracted with hot methanol. From band II compound FL-3, m.p. 66°C (400 mg) was isolated. From bands I, III, IV, & V no compound could be isolated in sufficient amount.

SECTION – I

Characterisation of Compound FL-1

2'-hydroxyisopropyldihydrofuranocoumarin

The white shining compound FL-1 , m.p. 189°C, was analysed for $C_{14}H_{14}O_4$ on the basis of elemental analysis and molecular weight determination (M^+ 246).

Compound gave blue-violet fluorescence under UV light[1] .

The UV absorption maxima at 225, 250, 260, 300 (sh) and 337 nm was specific for dihydrofuranocoumarin[2].

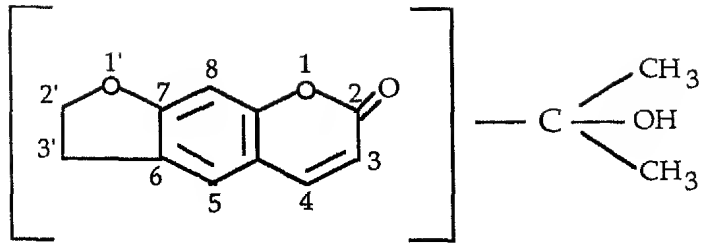
Appearance of peaks at 3440 cm^{-1} and 1700 cm^{-1} suggested the presence of tertiary hydroxyl group and α,β - unsaturated δ - lactone, respectively.

The hydroxyl group was found to be alcoholic since it gave a negative test with ferric chloride . The compound gave no product with acetic anhydride and sodium acetate at room temperature but on heating the reaction mixture in an oil-bath at 130°-140°C, mono-acetate of the compound was formed which confirmed the presence of tertiary hydroxyl group in the molecule.

The compound did not contain methoxyl and methylenedioxy group as suggested by ^1H NMR data and IR spectrum.

Spectral studies of the compound suggested the presence of a hydroxyisopropyl side chain. The ^1H NMR signals at δ 1.22 (s, 3H, CH_3), 1.36 (s, 3H, -CH_3) and 2.20 (bs, 1H, -OH) ppm confirmed the presence of hydroxyisopropyl side chain[3]. The fragment at m/z 59 also confirmed the presence of hydroxyisopropyl side chain[4].

Thus, ^1H NMR spectrum of the compound was consistent with dihydrofuranocoumarin containing a hydroxyisopropyl substituent and could be represented as I :

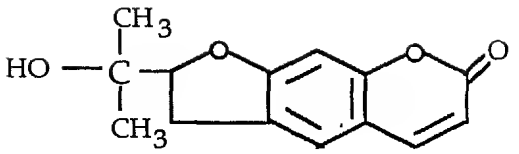


(I)

Signals at δ 6.20 (d, 1H, J = 9Hz) and 7.60 (d, 1H, J= 9Hz) ppm were assignable to the protons at C-3 and C-4[4]. The ^{1}H NMR spectrum also revealed the presence of two singlets, one at δ 7.22 (1H) and the other at δ 6.74 (1H) ppm assignable to C-5 and C-8 protons, respectively[5].

The only thing left in the structure of the compound FL-4 was the position of the side chain, present either at C-2' or C-3' . This was confirmed on the basis of ^{1}H NMR spectrum. The methylene and methine protons on the dihydrofuran ring constitute an A_2X system which appears as a well-defined two proton doublet at δ 3.20 (J = 9Hz) and a one proton triplet at δ 4.74 (J = 9 and 9Hz) ppm[6]. It showed that the hydroxyisopropyl side chain was present at C-2' position. If the side chain were present at C-3' position then a doublet corresponding to two protons should be present at δ 3.75 ppm or at higher value due to the deshielding effect of the neighbouring oxygen atom.

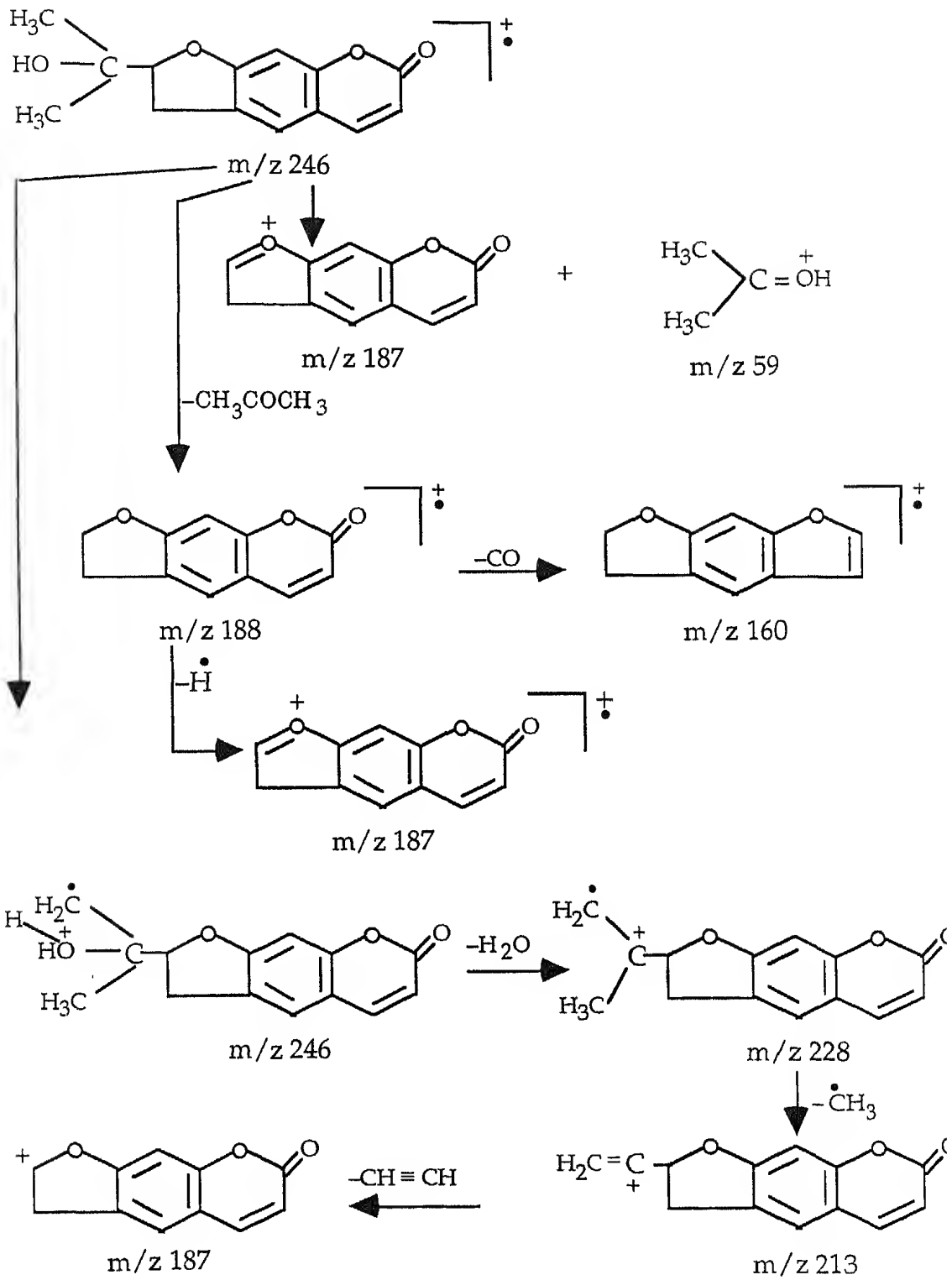
On the basis of above evidences, the compound, 2'-hydroxyisopropyldihydrofuranocoumarin, is represented as II :



(II)

Finally, the structure of II as 2'-hydroxyisopropyl-dihydrofuranocoumarin was confirmed by mass spectral data. The mass spectrum of the compound showed the molecular ion peak at m/z 246. The principal fragmentation pattern of hydroxyisopropyl dihydrofuranocoumarin, involves the loss of acetone from the molecular ion by elimination of the hydroxylated side chain with rearrangement of a hydrogen atom to give the ion, m/z 188[7]. This then losses a hydrogen atom to give a highly stabilized ion of m/z 187 as a base peak. Fission of the hydroxyisopropyl side chain with retention of the charge on the smaller fragment to give an abundant ion at m/z 59, due to the protonated form of acetone[4], was also a characteristic feature of such coumarin. The fragmentation pattern has been represented in scheme I.

Scheme I


m/z 246
m/z 187
m/z 59
-CH3COCH3
m/z 188
-CO
m/z 160
-H
m/z 187
-H2O
m/z 246
m/z 228
-CH3
-CH≡CH
m/z 187
m/z 213

The compound FL-1 as marmesin was confirmed by its m.p., mmp. and co-chromatography with an authentic sample of marmesin[8].

Solubility	:	Chloroform, benzene, acetone and methanol	
m.p.	:	189°C	
R_f	:	0.65 (ethyl acetate)	
Elemental Analysis	:	Found	Calculated for $C_{14}H_{14}O_4$
		C : 68.54%	C : 68.30%
		H : 5.70%	H : 5.69%
UV λ_{max}^{MeOH}	:	225, 250, 260, 300 (sh), 337 nm.	
IR ν_{max}^{KBr}	:	3440, 3000, 1700, 1628, 1565, 1485, 1445, 1362, 1128, 960, 840 cm^{-1}.	

^1H NMR ($CDCl_3$, 90 MHz) :

Assignments	Chemical shifts (δ - ppm)	*J* values (Hz)
$-CH_3$	1.22 (s, 3H)	
$-CH_3$	1.36 (s, 3H)	
Ar-CH_2-CH<	3.20 (d, 2H)	9
Ar-CH_2-CH<	4.74(t,1H)	9,9
H-3	6.20 (d, 1H)	9
H-8	6.74(s, 1H)	
H-5	7.22 (s, 1H)	
H-4	7.60 (d,1H)	9
- OH	2.20(bs,1H)	

Mass spectra,m/z : M^+ 246, 228, 213, 188, 187, 166.

Acetylation of compound FL-1

The compound (0.05g) was acetylated with acetic anhydride (4ml) and sodium acetate (1g). The mixture was taken in a 100 ml round bottom flask and refluxed for 4 hr in an oil bath at 130-140°C . It was cooled and added to ice-cold water (150 ml). The precipitate obtained was filtered, washed several times with water and dried. It was crystallised from methanol as white crystalline compound.

SECTION - II

Characterisation of Compound FL-2

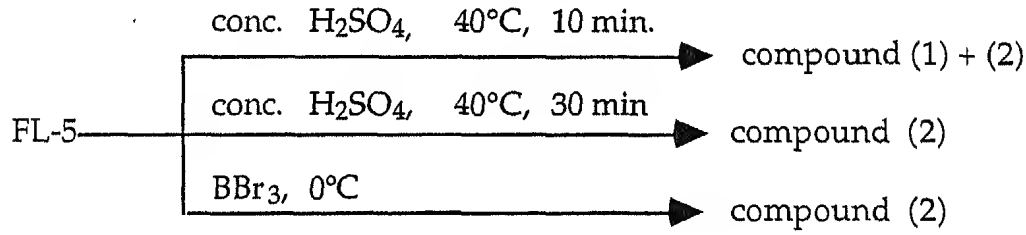
Fernolin

The compound FL-2, m.p.262°C was analysed for $C_{22}H_{20}O_7$ on the basis of molecular weight determination (M^+ 396). The compound gave blue fluorescence under UV light[1].

The linear furanocoumarin nature of compound was confirmed by its UV absorption maxima at 220, 252, 276 and 312 nm, as furanocoumarins absorb in the same region[9].

IR absorptions at 1755 and 1710 cm^{-1} indicated the presence of α,β-unsaturated γ-lactone and α,β-unsaturated δ–lactone[10]. The presence of methoxyl and methyl groups was inferred by bands at 2920 and 2800 cm^{-1} in its IR spectrum..

On treatment with conc. H_2SO_4 for 10 minutes at 40°C, it afforded compound (1) as a major product whereas for 30 minutes compound (2) was the only product and on treatment with BBr_3 at 0° the compound FL-2 afforded only compound (2).

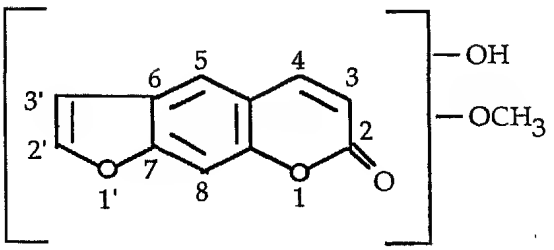


Compound (1)

The UV absorption maxima of the compound (1) at 220, 242 (sh), 250 and 314 nm was in close agreement with that reported for 5,8-dioxygenatedfuranocoumarin.[11]

The ^1H NMR spectrum revealed the presence of one methoxyl [δ 4.25 ppm (s, 3H)] and one hydroxyl [δ 6.20 ppm (s,-OH)] function.

Compound (1) can, thus, be represented as III :

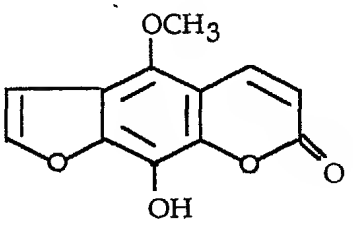


(III)

Four signals corresponding to one proton, were present in the aromatic region of the 1 suggesting the disubstituted furanocoumarin.

A pair of doublets at δ 6.30 and 7.76 ppm (J = 9 Hz) in its ^1H NMR spectrum was attributed to the pyran ring protons at C-3 and C-4, respectively. Another pair of doublets appeared at δ 7.68 and 6.81 ppm, (J = 2.3 Hz) corresponding to 2' - and 3'-furan ring protons. A sharp singlet at δ 4.25 ppm corresponded to methoxyl group which was present at C-5 position. So, the position left for hydroxyl group was C-8 position.

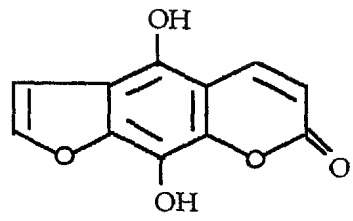
Thus, the structure assigned to the compound (1), 5-methoxy-8-hydroxyfuranocoumarin, has been represented as IV :



(IV)

The structure of 1 was also confirmed by its m.p. (214°C) m.p. and co-chromatography with an authentic sample[12]. Compound (2) was found to be 5,8–dihydroxyfuranocoumarin on direct comparison

with an authentic sample[12] (m.p. 210°C). Structure of 2 could, thus, be represented as V:



(V)

On the basis of above evidences, it is clear that the compound (1) was present as the nucleus in the compound FL-2 or in other word, the compound FL-2 was ether derivative of 1.

The 200 MHz spectrum of the compound FL-2 integrated for 20 protons and assignments of their chemical shifts are given in Table I.

Table - I

Assignments	**Chemical Shifts (δ–ppm)**	***J* values (Hz)**
H-2'	7.71 (d, 1H)	2.3
H-3'	6.80 (d,1H)	2.3
H-3	6.35 (d, 1H)	9.5
H-4	7.76 (d, 1H)	9.5
H-1"	5.0 (d, 2H)	6.6
H-2"	5.72 (tm, 1H)	6.6 & 0.98
H-4"	1.79 (brs, 3H)	

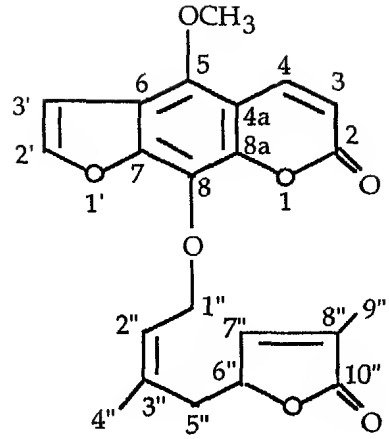
H-5"	2.36 (m, 2H)	6.5
H-6"	4.92 (tm,1H)	6.5 & 1.95
H-7"	6.93 (dq, 1H)	1.71 & 0.16
H-9"	1.88 (t, 3H)	1.71 & 1.95
$-OCH_3$	3.90 (s, 3H)	

A pair of doublets of δ 6.35 (1H, J = 9.5 Hz) and 7.76 (1H, J = 9.5 Hz) ppm was assigned to the C-3 and C-4 protons of the coumarin nucleus. Such doublets were characteristic of the unsubstituted pyrone nucleus. Another characteristic pair of doublets for the protons C-2' and C-3' appeared at 7.71 (1H, J = 2.3 Hz) and 6.80 (1H, J=2.3Hz)ppm. A doublet of two protons which appeared at δ 5.09 (J = 6.6 Hz) was assigned to the $-OCH_2-$ group at C-1". The olefinic proton at C-2" appeared as multiplet at δ 5.72 ppm (J=6.6 and 0.98 Hz) due to the coupling effect of methylene and methyl protons at C-1" and C-4", respectively. The three methyl protons at C-4" appeared as broad singlet at δ 1.79 and the methylene group at C-5" appeared as multiplet at δ 2.36 ppm. The multiple centered at δ 4.92 ppm (J = 6.5 and 1.95 Hz) was assigned to the protons at C-6" of a five-membered lactone. Signal for H-7" appeared as multiplet at δ 6.93 ppm (J = 1.71 and 0.16 Hz) coupled by H-6" (methine proton) and H-9" (methyl proton). The triplet at δ 1.88 ppm (J = 1.71 and 1.95 Hz) for three protons could be assigned to the methyl function at the α-position of the α, β-unsaturated γ-lactone group. The multiplicity being

explained by the assumption of the long range homo-allylic with H-6".

The structure of this compound was confirmed by decoupling experiment (NMDR technique). The relationship of H-9" with H-6" and H-7" was established by decoupling experiment. Irradiation at δ 1.8866 ppm caused the multiplet (dq at δ6.93, H-7") change to a doublet accompanied by a simultaneous change in the shape of the multiplet (tm at δ 4.92, H-6"). Irradiation at δ 2.3648 simplified the multiplets at δ 4.92 (H-6") and 6.93 (H-7") establishing the relationship of H-5", H-6" and H-7". This was further supported by the appearance of sharp singlet at δ 1.88 ppm (H-9"), a dd at δ2.36 (H-5") and simplification of the multiplet at δ 6.93 (H-7") by irradiation at δ 4.9254. Irradiation at δ 5.0416 simplified the multiplet at δ5.72 (H-2") showing the relationship between H-1" and H-2" as supported by the change of doublet at δ 5.09 (H-1") on irradiation at δ5.7267. Irradiation at δ6.9342 changed the double doublet appearing like the triplet at δ 1.88 (H-9") to doublet accompanied by simultaneous change in the shape of multiplet at δ4.92 (H-6").

On the basis of above findings, the structure of the compound FL-2 as fernolin has been represented as (VI)



(VI)

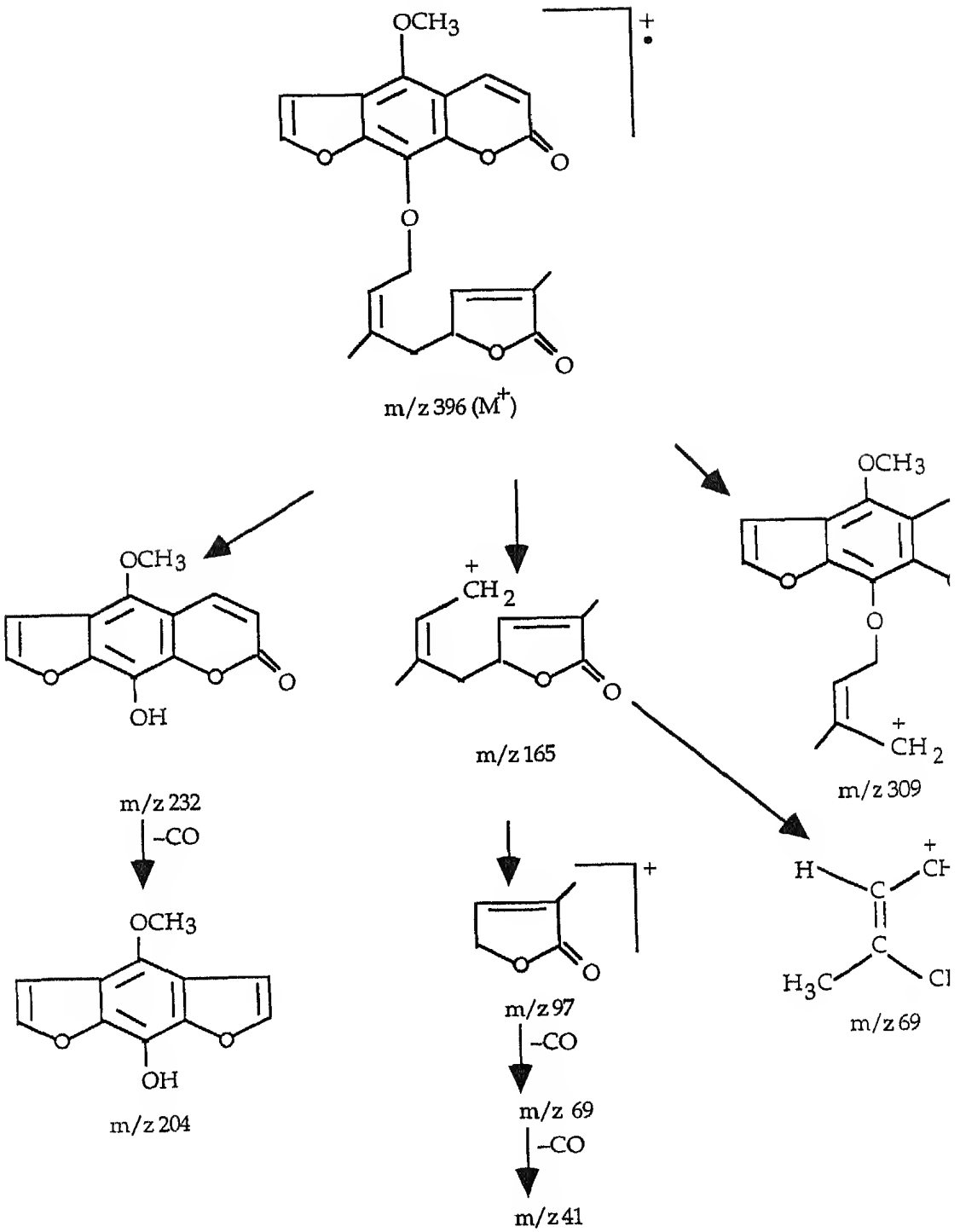
The structure of the compound FL-2 as VI was also confirmed by ^{13}C NMR data[10].

The ^{13}C NMR of the compound showed 21 signals for 22 carbons. The signals at δ 10.58 and 17.24 ppm were assigned to the methyl carbon at C-4" and C-9", respectively. The signal at δ 43.37 could be assigned to the methylene carbon (C-5") while another $-CH_2-$carbon (C-1") appeared appreciably downfield at δ69.69 ppm showing its linkage with oxygen. Similarly, another saturated carbon C-6" appeared at δ79.54 ppm because of the adjacent oxygen atom (ether linkage). The signals at δ106.85 and 146.70 were assigned to C-3' and C-2', respectively. The two unsubstituted carbon in the coumarin ring appearing at δ 114.76 (C-3) and 144.32 (C-4) shifted to a doublet by OFR (off field resonance) experiment. Ring carbons appearing at δ 116.58 (C-4a), 148.65 (C-8a), 125.95 (C-6), 148.26 (C-7) and 131.48 (C-8) did not show coupling, which confirmed the absence of proton on them. Aromatic carbon at position C-5 appearing at δ131

ppm also showed no doublet with OFR, confirming that position -5 was substituted. Peaks for C-5, C-7, C-8 and C-8a appeared downfield as compared to C-4a and C-6 because of the oxygenation at C-5, C-7, C-8 and C-8a. Olefinic carbons appearing at δ 123.94 and 137.02 were assigned to C-2" and C-3". Signal for C-7" appearing together with C-4 at δ144.32 also showed a doublet by OFR experiment. The two-signals for low intensity at δ160.37 and 173.82 were assigned to the carbonyl carbons, C-2, and C-10", respectively. Signal for C-8" appeared at δ130.8 ppm. A signal appeared at δ63.00 for methoxyl group at C-5.

The size and the nature of the side chain was assessed by the study of its mass spectrum. Mass spectral data showed base peak at m/z 232 and molecular ion peak at m/z 396. Appearance of the ions at m/z 309 and 165 were diagnostic of the side chain attached to oxygen at C-8 of the furocoumarin nucleus. The presence of a base peak at m/z 232 and ion at m/z 204 provided sufficient evidence for the presence of furanocoumarin nucleus. The ion at m/z 97 was attributable to γ-lactone moiety of the side chain. The fragmentation pattern has been represented in shceme II.

Scheme - II


OCH3
O
O
O
m/z 396 (M+)
OCH3
OH
m/z 232
-CO
OCH3
OH
m/z 204
+
CH2
O
m/z 165
OCH3
O
+
CH2
m/z 309
+
O
m/z 97
-CO
m/z 69
-CO
m/z 41
H
C
C
H3C
Cl
m/z 69

m.p.	:	262°C	
Elemental analysis	:	Found	Calculated for $C_{22}H_{20}O_7$
		C : 65.26%	C : 66.66%
		H : 5.01%	H : 5.05 %
UV λ_{max}^{MeOH}	:	220, 252, 276, 312 nm.	
IR ν_{max}^{KBr}	:	2920, 2800, 1755, 1710, 1615, 1590, 1440, 1400, 1325, 1290, 1210, 1180, 1150, 1025 cm^{-1}	
^{13}C NMR ($CDCl_3$)	:	δ 160.37 (C-2), 114.76 (C-3), 144.32 (C-4 & C-7"), 116.58 (C-4a), 131.0, (C-5), 125.95 (C-6), 148.26 (C-7), 131.48 (C-8), 148.65 (C-8a), 146.70 (C-2'), 106.85 (C-3'), 69.69 (C-1"), 123.94 (C-2"), 137.02 (C-3"), 10.58 (C-4"), 43.37 (C-5"), 79.54 (C-6"), 130.8 (C-8"), 17.24 (C-9"), 173.82 (C-10"), 63.00 (-OCH_3) ppm.	
Mass spectra, m/z	:	396 (M^+), 309, 232, 204, 165, 97, 69, 41.	

Dealkylation of compound FL-2

Method 1

To the compound (0.1g) dissolved in acetic acid (1 ml), two drops of conc. H_2SO_4 were added. The reaction mixture was heated

for 10 min (40°C), cooled, diluted with ice water and extracted with ethyl acetate. The ethyl acetate part was washed with water, dried over anhydrous Na_2SO_4 and the solvent removed to afford the crude products. Purification over preparative TLC on silica gel yielded 5-methoxy-8-hydroxyfuranocoumarin, compound (1), m.p. 214°C and 5,8-dihydroxyfuranocoumarin, compound (2) , m.p. 210°C.

When the reaction mixture was heated for 30 min (40°C) and worked up as usual, only compound (2) was obtained, m.p. 210°C.

Method 2

To a well-stirred solution of the compound (0.05g) in dichloromethane (20 ml), borontribromide (0.05g) in dichloromethane (10 ml) was added at 0°C . The stirring was further continued at room temperature for 24 hr. The solution was then poured into water and extracted with ethyl acetate. The crude product on crystallisation gave a crystalline product, 5,8-dihydroxyfurano coumarin, m.p. 209°C.

Compound (1)

m.p. : 214°C

UV λ_{max}^{MeOH} : 220, 242 (sh), 250, 314 nm.

^1H NMR ($CDCl_3$, 90 MHz) :

Assignments	**Chemical shifts (δ - ppm)**	***J* values (Hz)**
-OH	6.20 (s, 1H)	
$-OCH_3$	4.25 (s, 3H)	
H-3	6.30 (d, 1H)	9
H-3'	6.81 (d,1H)	2.3
H-2'	7.68 (d, 1H)	2.3
H-4	7.76 (d,1H)	9

SECTION - III

Characterisation of compound FL-3

7-geranyloxycoumarin

The light yellow coloured shining crystal, m.p. 66°C, was analysed for $C_{19}H_{22}O_3$ on the basis of elemental analysis and molecular weight determination.

The UV spectrum displayed absorptions at 245, 254, and 324 nm, similar to that of monoalkoxycoumarin[13].

The IR spectrum of the compound suggested the presence of α, β-unsaturated δ-lactone (1725 cm^{-1})[14], gem-dimethyl group (2900-3000 cm^{-1}) and the aromatic ring (1610 cm^{-1}).

Compound FL-3 as 7-alkoxycoumarin was confirmed by ^1H NMR spectrum. ^1H NMR spectrum of the compound showed the presence of five aromatic protons. This means that the coumarin nucleus in monosubstituted . The ^1H NMR data of the compound FL-3 are given in table I.

Table - I

^1H NMR ($CDCl_3$, 60MH_z) :

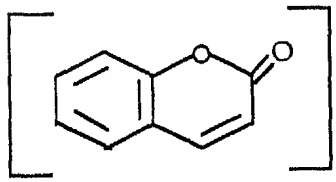
Assignments	**Chemical shifts (δ - ppm)**	***J* values (Hz)**
CH_3 C CH_3	1.6 & 1.65 (s, 3H each)	
3'-Me	1.75 (s, 3H)	
4'->CH_2 & 5'-> CH_2	2.0-2.20 (m, 4H)	
1'-> CH_2	4.60 (d, 2H)	6.5
6'-> CH_2	5-5.2 (bm, 1H)	
2'>CH	5.48 (t, 1H)	6.8

H-3	6.25 (d, 1H)	9.5
H-6 & H-8	6.85 (m, 2H)	
H-5	7.37 (d, 1H)	8.5
H-4	7.65 (d, 1H)	9.5

It showed the presence of either geranyloxy or neryloxy side chain[15]. The structure of the side chain was established on the basis of ^{1}H NMR spectrum.

^{1}H NMR spectrum of the coumarin displayed signals at δ 1.60 and 1.65 (s, 3H each, gem-dimethyl group at 7'), 1.75 (s, 3H, 3'-Me), 2.0-2.20 (m, 4H, 4'->CH_2 and 5'->CH_2), 4.60 (d, 2H, J = 6.5 Hz, 1' - > CH_2), 5-5.2 (bm, 1H, 6'- > CH coupling with vicinal 5'->CH_2 and allylic 7'-Me's) and 5.48 (t, 1H, J = 6.5 Hz, 2'-> CH coupling with 1'-CH_2 and also with allylic 4'-> CH_2 and 3'-Me) for either geranyloxy or neryloxy side chain.

The structure of the compound, thus, could be represented as I :



– geranyloxy or neryloxy side chain

(I)

It's ^{1}H NMR spectrum also revealed the presence of two doublets at δ 6.25 (d,1H, J = 9.5 Hz) and 7.65 (d, 1H, J = 9.5 Hz) ppm assignable to C-3 and C-4 protons, respectively. A doublet at δ 7.37 ppm (1H, J = 8.5 Hz) clearly indicated the presence of a proton at C-5.

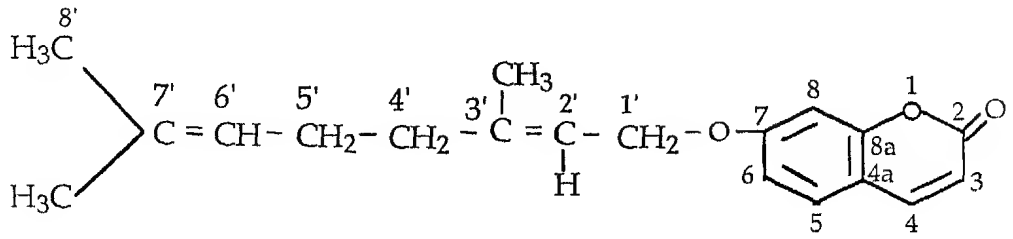
Due to the overlapping of the signals of C-6 and C-8 protons, a multiplet corresponding to two protons at δ 6.85 ppm has been observed[11]. Thus, it clearly indicated that the side chain was located at the position C-7, in the coumarin nucleus[4].

The nature and the position of the side chain was evident by the study of high resolution ^{1}H NMR spectrum[16]. The geometry about the double bond (2'-3'position) has been suggested to be trans (geranyloxy form) rather than cis (neryloxy form)[17], on the basis of fine spitting of the 2'-vinyl hydrogen in the ^{1}H NMR spectrum. At 60 MHz, the vinyl proton at C-2' appeared as a triplet at δ 5.48 ppm (1H, J = 6.8 Hz)[18]. But at 300 MHz, this absorption appeared as a triplet (J = 6.8 Hz) with each peak further splitting into quartets (J = 1.2 Hz). This splitting and coupling constant along with stereochemical considerations and the reported trans arrangement of the -Me group to hydrogen in geraniol[19], suggested that the vinyl proton was trans to the -Me group.

In ^{1}H NMR spectrum at 300 MHz, the signal for C-6 proton ortho-coupled to C-5 proton and meta-coupled to C-8 proton, appeared as a double doublet at δ 6.85 ppm (1H, J =8.5 and 2.4 Hz) and the signal for C-8 proton which was only meta-coupled to C-6 proton, appeared as a doublet at δ 6.82 ppm (1H, J = 2.4 Hz).

The above high resolution ^{1}H NMR data confirmed the presence of a geranyloxy side chain at C-7 position of the coumarin nucleus.

The structure of the compound can, thus, be represented as 7-geranyloxycoumarin (II) :



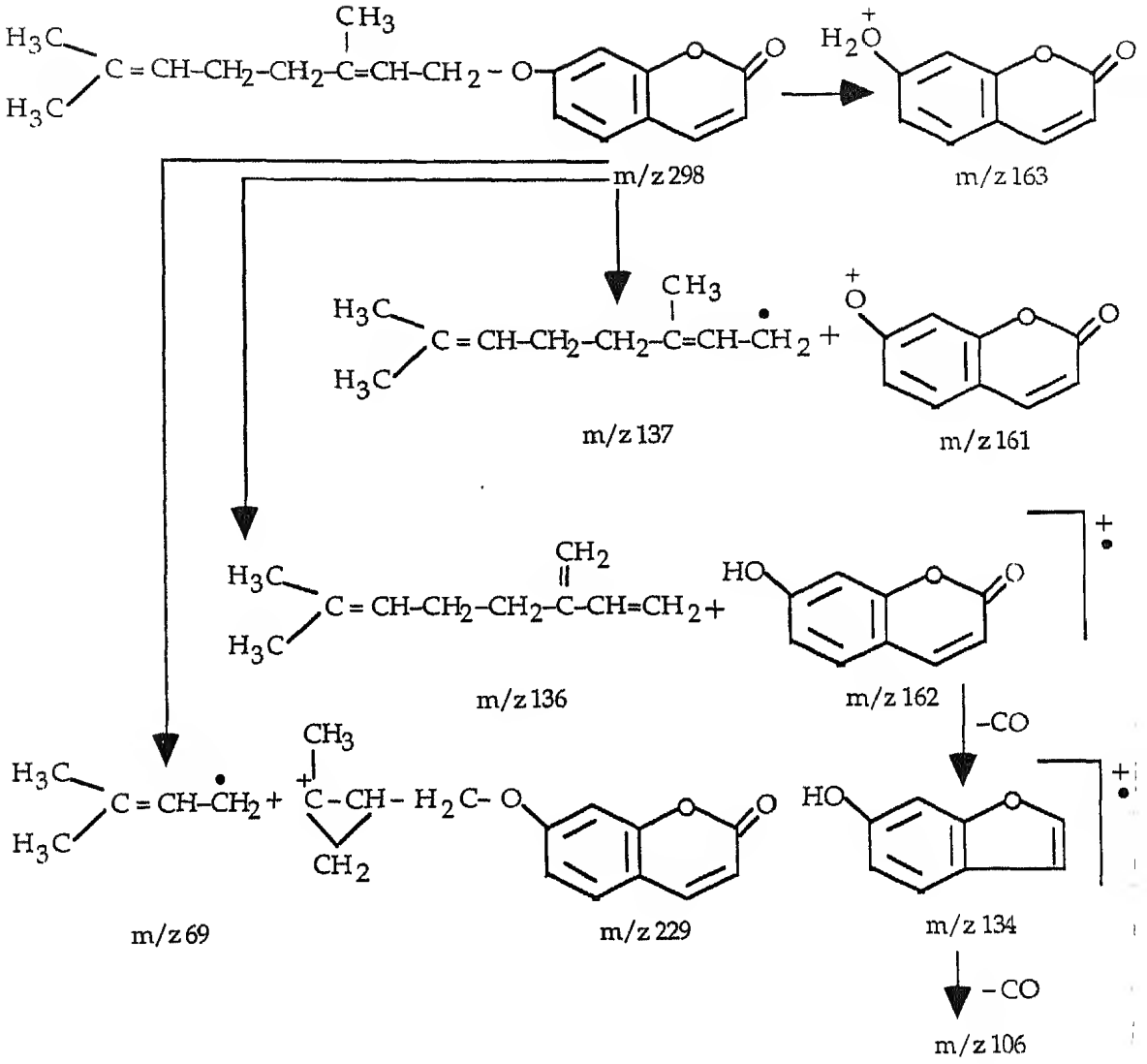
(II)

The structure of the compound FL-3 as 7-geranyloxycoumarin was further confirmed by ^{13}C NMR data[20]. The chemical shifts of all the carbons of coumarin nuclei of 7-geranyloxycoumarin were very much similar to those of 7-hydroxycoumarin[21]. The signal for C-7, when compared with that of simple coumarin, appeared appreciably downfield (about 30 ppm), indicating its linkage with oxygen function and the signals for C-6 and C-8 appeared upfield. The effect on C-5 and C-8a (meta-carbons) was not much, usually 0.5-2 ppm downfield. The chemical shifts of the side chain carbons of 7-geranyloxycoumarin were very similar to those of geraniol[22] except C-1' , C-2' and C-3'. C-1' and C-3' underwent slight downfield shift while C-2' underwent upfield shift. The assignments of the chemical shifts of all the carbons are given in experimental section.

The mass spectrum exhibited molecular ion peak at m/z 298. The mass spectrum was consistent with structure II and was characterised by the extremely facile fragmentation of the allylic ether bond with hydrogen transfer from the 3'-Me to the ether oxygen in a 6-membered cyclic transition state, resulting in a 7-

hydroxycoumarin radical ion, appearing as a base peak at m/z 162 and consequently, the parent ion at m/z 298 was extremely weak[23-24]. The other diagnostically important peaks were at m/z 229 (M-69, 4'-5' cleavage), 163 (1'-O cleavage with intra-molecular capture of 2H), 161 (M-137, 1'-O cleavage), 134 (a-CO to give benzofuran type ion), 136 and 137 (side chain after 1'-O cleavage , with or without H-loss to the ether oxygen), 106 (loss of CO from 134), 68 and 69 (5'-4' cleavage with or without H-loss). Fragmentation pattern has been represented in scheme I.

Scheme - I



The compound FL-3 was confirmed as 7-geranyloxycoumarin (aurapten) by direct comparison (m.p., mmp, Co-TLC) with an authentic sample[20].

EXPERIMENTAL

Solubility : Benzene, chloroform, acetone and methanol

Chromatography : Preparative TLC was carried on silica gel'g' plates using following solvent system.

Benzene : Ethyl acetate (9:1, v/v) (Rf = 0.90).

m.p. : 66°C

R_f : 0.90 (benzene : ethyl actate, 9 : 1, v/v)

Elemental analysis :

Found C : 75.91%, H : 6.01 %

Calculated for $C_{19}H_{22}O_3$ C : 75.82%, H : 6.3%

Spectral studies

UV λ_{max}^{MeOH} : 245, 254, 324 nm.

IR ν_{max}^{KBr} : 3000-3100, 2900-3000, 1725, 1610 , 1400, 1350, 1235, 1200, 1125 cm^{-1}.

^1H NMR [$CDCl_3$, 300 MH_2] : δ 1.6 and 1.65 (s, 3H each, $\overset{CH_3}{CH_3}$ > C-), 1.76 (s, 3H, 3'-CH_3), 2.0 - 2.20 (m, 4H, 4'->CH_2 and 5'- > CH_2), 4.60 (d, 2H, J = 6.5 Hz, 1' - > CH_2), 5-5.2 (bm, 1H, 6' - > CH), 5.48 (tq, 1H, J = 6.8 and 1.2 Hz, 2' - > CH), 6.25 (d, 1H, J = 9.5 Hz, H-3), 6.82 (d, 1H, J = 2.4 Hz, H-8), 6.85 (dd, 1H, J = 8.5 and 2.4 Hz, H-6), 7.37 (d, 1H, J = 8.5 , H-5) and 7.65 (d, 1H, J = 9.5 Hz, H-4) ppm.

^{13}C NMR [$CDCl_3$]	:	δ 161.2 (C-2), 112.4 (C-3), 143. 4 (C-4), 112.3 (C-4a), 128.6 (C-5), 113.2 (C-6), 162.2 (C-7), 101.6 (C-8), 155.9 (C-8a), 65.5 (C-1'), 118.4 (C-2'), 142.3 (C-3'), 39.5 (C-4'), 26.2 (C-5'), 123.6 (C-6'), 131.9 (C-7'), 25.6 (C-8'), 17.7 (3'-Me), 16.7 (7'-CH_3) ppm.
Mass spectra, m/z	:	M^+ 298, 229, 163, 162, 161, 137, 136, 106, 69, 68.

SECTION - IV

Characterisation of Compound FL-4

Feronolide

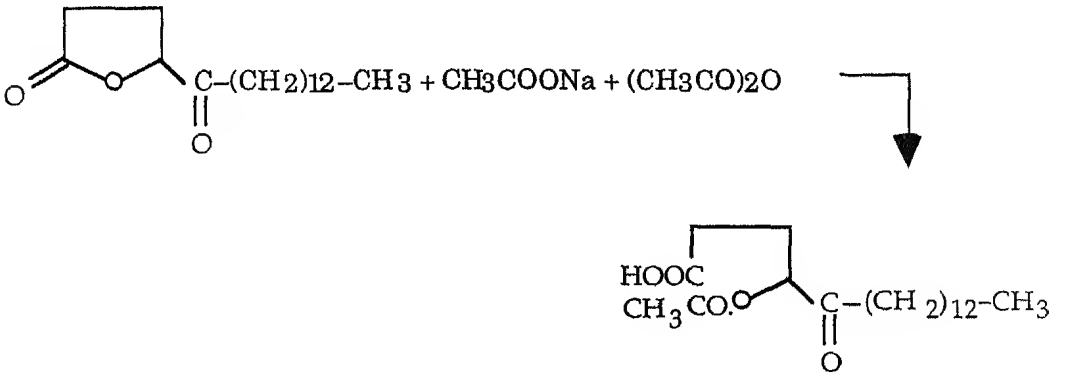
The creamish white coloured amorphous compound, m.p. 115°C on the basis of elemental analysis was found to have molecular formula $C_{18}H_{32}O_3$ [M+296].

The compound did not decolourise $KMnO_4$ solution and bromine water showed that compound is saturated . The compound was not burnt with smooky flame confirms aliphatic nature of compound.

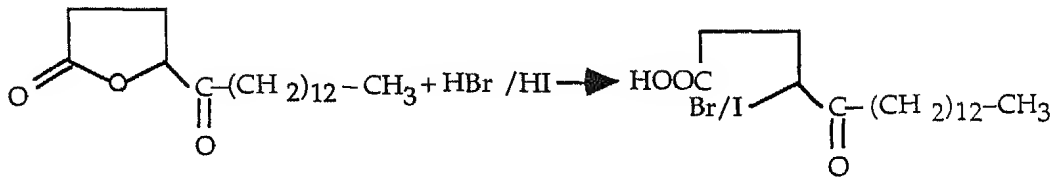
The peak at 1754 cm^{-1} in the IR spectra showed that the compound was a lactone[25-30] and the peaks at 1480 cm-1[31] and 1136 cm-1[32,33] further confirmed that the compound was an aliphatic ketolactone. On treatment with 2:4 dinitrophenyl hydrazine solution it gave a crystalline 2:4 dinitrophenyl hydrazone further confirming the presence of carbonyl group.

The structure of the compound has been further confirmed by following chemical reactions.

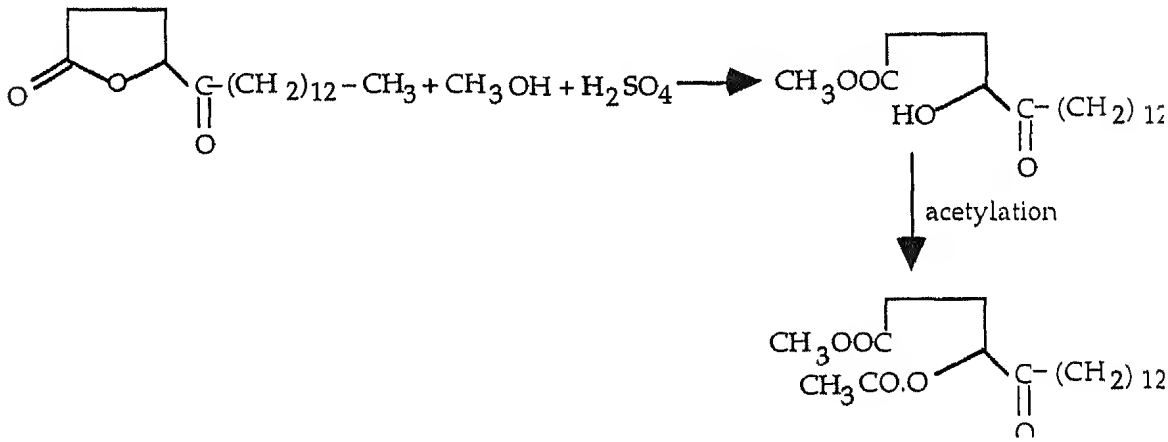
When compound refluxed with acetic anhydride and sodium acetate, the lactone ring opens up due to presence of sodium acetate and the hydroxyl group so formed gets acetylated by acetic anhydride.



On boiling with HBr and HI the lactone ring opens with the formation of bromo or iodo carboxylic acid having the following structure :

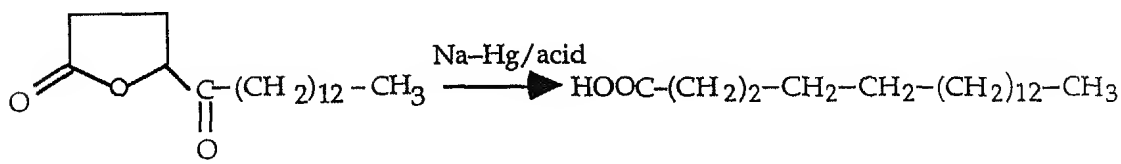


Similarly on esterification with methanol and concentrated H_2SO_4, it gives :



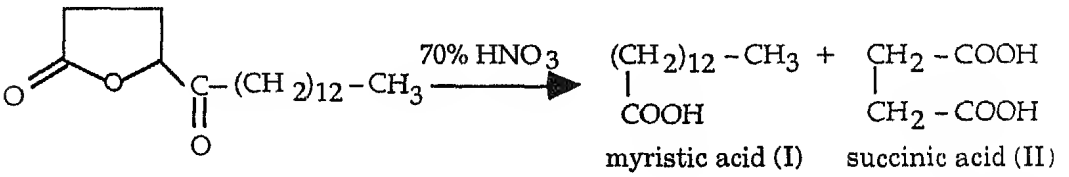
All these reactions confirming the lactone nature of the compound.

The appearance of the absorption bands at 758 cm^{-1} indicated the presence of long aliphatic chain in compound. The number of carbon atoms in the compound was confirmed by the reduction of compound with sodium amalgam and mineral acid.



This reaction indicates that the formed fatty acid was found to be identical with stearic acid. This shows that the compound was made up of straight chain of eighteen carbon atoms.

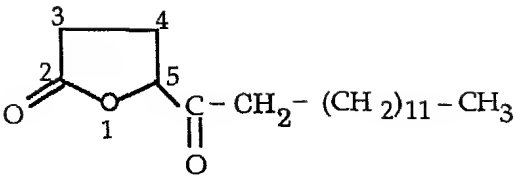
When the compound was undergo oxidation with 70% HNO_3, the mixture of two acids i.e. $C_{14}H_{28}O_2$ and $C_4H_6O_4$ were isolated.



The formation of these two acids confirmed that the compound have ϒ lactone ring and side chain is of 14 carbon atoms.

^1H NMR spectrum of the compound showed the singlet at δ 1.22 integrating 22 protons confirmed the presence of 11 methylene units. The compound showed the presence of terminal methyl group as triplet at δ 0.88. A two proton triplet appearig at δ 2.30 was attributed to one methylene unit attached to the carbonyl carbon atom. The triplet at δ 4.20 was assigned to the proton at C-5 of a ϒ lactone ring. The triplet at δ 4.5 and quartet at δ 2.5 for two protons could be assigned to the methylene group of lactone ring at C-3 and C-4, respectively.

The above observations clearly indicated that the following structure is being proposed for the compound FL-4 named as feronolide.

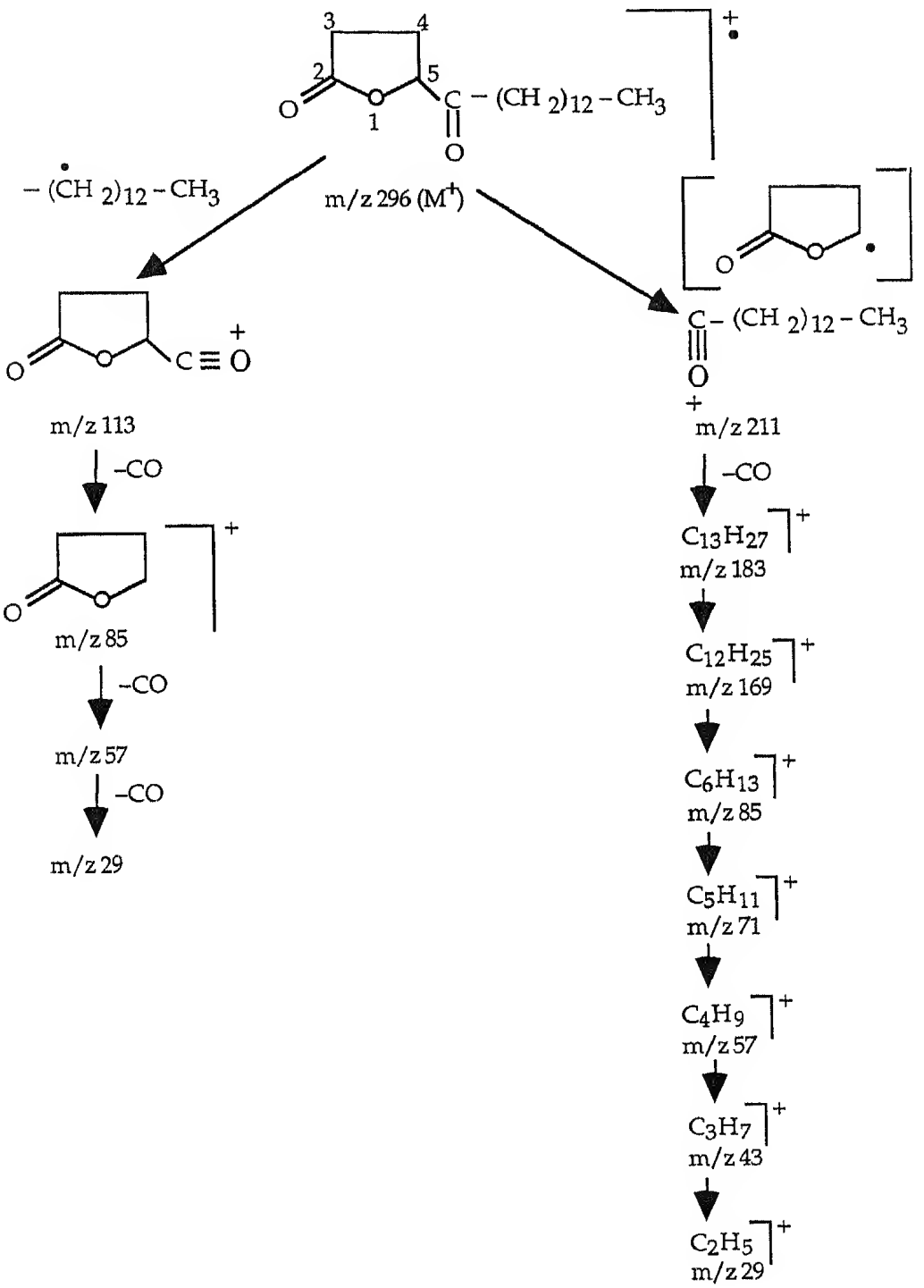


Feronolide

This structure of compound has supported by its mass fragmentation pattern .

Mass spectrum of compound

The mass spectrum exhibited molecular ion peak at m/z 296.



It was crystallised from ethanol.

Solubility	:	Chloropform , hot benzene, methanol and ethanol	
R_f	:	0.41	
Elemental Analysis	:	Found	Calculated for $C_{18}H_{32}O_3$
		C : 72.45%	C : 72.9%
		H : 11.2 %	C : 10.8%
IR ν_{max}^{KBr}	:	2934 (C-H streching), 1754 (lactone), 1480 (C=O group), 1136 (aliphatic ketone), 758 (aliphatic chain) cm^{-1}.	
^1H NMR	:	δ 0.88 (3H, t, terminal methyl group),	
[$CDCl_3$, 60Mz]		1.22 (22H, br s, 11 x CH_2), 2.30 (2H,t,-$COCH_2$-), 4.20 (1 H, t, C-5), 2.5 (2H, q, C-4), 4.5 (2H, t, C-3) ppm.	
Mass spectra m/z	:	296 (M^+), 211, 183, 169, 113, 85, 71, 57, 43, 29.	

Formation of 2:4 dinitrophenylhydrazone of feronolide

0.35g of the compound and 0.5 g of 2:4 dinitrophenyl hydrazine were taken in a 50 ml pyrex conical flask and 10 ml of absolute ethanol were added. The contents of the flask were warmed. 5 ml of concentrated hydrochloric acid (A.R.) were added and the contents of the flask were heated to boiling to obtain a clear solution. The

contents of the flask were cooled overnight. 2:4 dinitrophenyl hydrazone separated was filtered at the pump and washed with cold ethanol and then with distilled water, dried in a vacuum desiccator over calcium chloride. It was recrystallised with ethanol acetone mixture when a yellow crystalline compound melting point 135°C was obtained. The m.p. of the hydrazone did not alter on subsequent crystallisations. The percentage of nitrogen was determined by semi-micro Duma's method[34].

Acetylation of compound FL - 4

0.35g of the compound with 0.5g of fused sodium acetate (A.R.) and 4 ml of acetic anhydride was taken in the flask and the mixture refluxed for about eight hours on sand bath. The contents of the flask after cooling were poured into a beaker containing 500 ml of ice cooled water, with constant stirring. It was kept over night. The product obtained after filteration was washed well with distilled water and dried. The product was crystallised from acetone and was found to melt at 62°C. The m.p. did not rise on further crystallisations. The percentage of acetyl group in acetylated product was determined by the method of Wiesenberger[35,36].

Action of hydrobromic acid / hydroiodic acid on compound FL-4

0.45g of the compound was refluxed with 10 ml of freshly distilled hydrobromic acid/hydroiodic acid in a 50 ml pyrex flask fitted with a quick-fit air condenser, on a sand bath for about ten hours. The contents of the flask after cooling were poured in a beaker

containing 250 ml of ice cold water with constant stirring. The precipitated mass was filtered and washed well with water, then with 2% solution of sodium thiosulphate to remove excess of bromine/iodine and finally with distilled water. It was crystallised from ethanol.

The percentage of bromine was determined by semi-micro catalytic combustion method[37].

Esterification of compound FL-4

0.55 g of feronolide was refluxed with 10 ml of methanol (A.R.) and 2 ml of concentrated sulphuric acid (A.R.) in a 50 ml pyrex flask with a quick-fit air condenser, on an electrical water bath for about 20 hr. Excess of methanol was distilled off from the reaction mixture and the resulting product was poured in distilled water . It was extracted with ether. The ethereal layer was washed well with distilled water till the washings were neutral to methylorange. Ether was distilled off partially and ethanol was added to it. The precipitated mass so obtained was filtered and dried over calcium chloride in a vacuum desiccator. The m.p. of the ester was found to be 62°C. It was crystallised from absolute ethanol, m.p. 62°C.

Reduction of compound FL - 4

0.45 g of feronolide and 35 ml of dilute HCl were taken in a 250 ml three necked flask fitted with a mechanical stirrer and a water condenser. The contents of the flask were heated on an electrical water bath. To the hot solution, about 1.5 g of sodium amalgam were

added in small quantities at regular intervals during two hours through the third neck which was closed with a stopper. The contents of the flask were further heated for about three hours with constant stirring. It was then allowed to cool over night and about 100 ml of cold distilled water were then added. The mixture was extracted with ether . The ethereal layer was washed well with distilled water till the washings were neutral to methylorange. The ethereal layer was dried over anhydrous sodium sulphate and ether was distilled off. The product obtained was dried in a vacuum desiccator over calcium chloride. It was crystallised from dilute acetone and the m.p. was found to be 69°C. The m.p. did not change on further crystallisation.

This was found to be an aliphatic acid. The IR spectra of the compound confirmed the presence of carboxyl group in the product and was identified to be stearic acid. The oxidation product showed no depression of m.p. on admixture with an authentic specimen of stearic acid[38].

Oxidation of compound FL - 4

0.75 g of the compound was taken in a pyrex conical flask with a quick fit air condenser and 15 ml of pure concentrated HNO_3(70%) was added to it. The contents of the flask were refluxed on an electric water bath for about ten hours. During refluxing brown fumes of nitrous oxide were evolved which subsided later on. On cooling, the reaction mixture was poured in about 100 ml of ice cold distilled water and kept in a refrigerator over night. The acid was obtained as

a waxy mass, oxidation product I. It was filtered at pump and washed with distilled water till the washings were neutral to methyl orange. The combined filtrate and the washings were evaporated to dryness. The residue was extracted with ether. The ether layer was washed with distilled water till the washings were neutral to methylorange. The ether layer was dried over anhydrous sodium sulphate and ether was distilled off. The white solid oxidation product II obtained was found to melt at 185°C. It was crystallised from hot water. The m.p. of the oxidation product was found to be 185°C . It was dibasic acid, gave flourescein test and was identified to be succinic acid by mixed melting point with authentic sample of succinic acid.

The waxy acid (I) obtained as above was crystallised from dilute acetone. The m.p. of the acid was found to be 54°C and the m.p. did not show any rise on subsequent crystallisation. The acid was optically inactive and did not react with 2 : 4 dinitrophenyl hydrazine to give the corresponding derivative. It was identified to be myristic acid. The melting point was not depressed when mixed with authentic sample of myristic acid.

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LIST OF PUBLICATIONS

1. Two New Triterpene Glycosides From *Dendrocalamus strictus, Pharmaceutical Biology,* **1999,** (accepted).

2. New Flavonoid Glycosides From *Cassia occidentalis, Indian Journal of Chemistry,* **1999,** (accepted).

3. Two Pentacyclic Triterpenes From *Calotropis procera, Indian Journal of Chemistry,* **2000,** (communicated).